

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

3

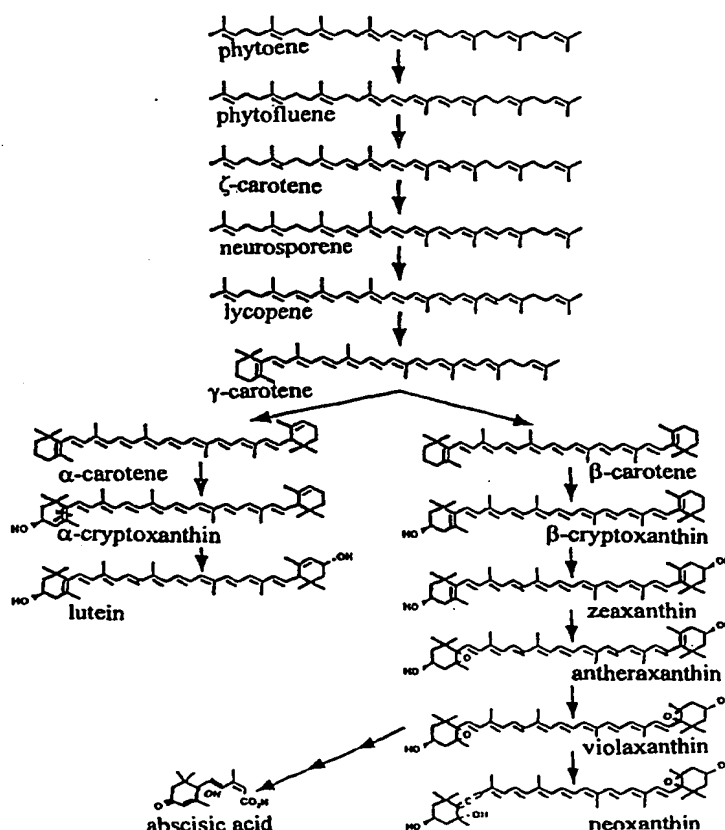
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 15/53, 15/61, 15/11, 9/02, 9/90, A01H 5/00, C12N 15/82, 5/10		A2	(11) International Publication Number: WO 00/32788
			(43) International Publication Date: 8 June 2000 (08.06.00)
(21) International Application Number: PCT/DK99/00668		(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), DM, EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR (Utility model), KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 30 November 1999 (30.11.99)			
(30) Priority Data: 09/201,641 30 November 1998 (30.11.98) US			
(71) Applicant (for all designated States except US): CHR. HANSEN A/S [DK/DK]; Boege Allé 10-12, P.O. Box 407, DK-2970 Hoersholm (DK).			
(72) Inventors; and (75) Inventors/Applicants (for US only): DELLAPENNA, Dean [US/US]; 4135 Longknife Road, Reno, NV 89557 (US). CUNNINGHAM, Francis, X. [US/US]; 2727 Washington Avenue, Chevy Chase, MD 20815 (US).			
(74) Agent: PLOUGMANN, VINGTOFT & PARTNERS A/S; Sankt Annæ Plads 11, P.O. Box 3007, DK-1021 Copenhagen K (DK).			
		Published Without international search report and to be republished upon receipt of that report.	

(54) Title: METHOD FOR REGULATING CAROTENOID BIOSYNTHESIS IN MARIGOLDS

(57) Abstract

A method for manipulating the ratio of various carotenoids in a plant as a means for augmenting the accumulation of selected carotenoids is described. Transgenic marigold plants which produce various ratios of carotenoids and methods for producing the same are described. Preferably, various carotenoids are accumulated in the petals of marigold by selecting a specific combination of isolated DNAs encoding various enzymes involved in the carotenoid biosynthesis pathway to produce antisense RNA, sense RNA or combinations thereof. Transgenic marigold which specifically accumulates carotenoids in the petals are described. Also described are isolated DNA sequences encoding the marigold genes beta-cyclase, epsilon-cyclase, beta-hydroxylase and isopentyl pyrophosphate isomerase.



BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon	KR	Republic of Korea	PL	Poland		
CN	China	KZ	Kazakhstan	PT	Portugal		
CU	Cuba	LC	Saint Lucia	RO	Romania		
CZ	Czech Republic	LI	Liechtenstein	RU	Russian Federation		
DE	Germany	LK	Sri Lanka	SD	Sudan		
DK	Denmark	LR	Liberia	SE	Sweden		
EE	Estonia			SG	Singapore		

METHOD FOR REGULATING CAROTENOID BIOSYNTHESIS IN MARIGOLDS

BACKGROUND OF THE INVENTION5 (1) Field of the Invention

The present invention provides a method for manipulating the ratio of various carotenoids in plants as a means for augmenting the accumulation of selected carotenoids. The present invention further relates to transgenic marigold plants which produce various ratios of carotenoids and methods for producing the same. Preferably, various carotenoids can be accumulated in the petals of marigold by selecting a specific combination of isolated DNAs encoding various enzymes involved in the carotenoid biosynthesis pathway to produce antisense RNA, sense RNA or combinations thereof. The present invention also describes isolated DNA sequences encoding the marigold genes beta-cyclase, epsilon-cyclase, beta-hydroxylase, isopentyl pyro-phosphate isomerase.

(2) Description of the Related Art

20 Carotenoids which comprise the most important group of 40-carbon terpenes and terpenoids are pigments that have a variety of commercial applications. Carotenoids are a class of hydrocarbons (carotenes) and their hydroxylated derivatives (xanthophylls) which comprise 40-carbon (C_{40}) terpenoids consisting of eight isoprenoid (C_5) units joined together. The terpenoids are joined in such a manner that the arrangement of the isoprenoid units is reversed at the center of the molecule placing the terminal methyl groups in a 1,6 relationship and the non-terminal methyl groups in a 1,5 relationship. Carotenoids can be monocyclic, bicyclic or acyclic. Carotenoids are produced by a wide variety of bacteria, fungi, and green plants. The carotenoids of the most value are intermediates in the carotenoid biosynthetic pathway and consist of lycopene (ψ, ψ -carotene), beta-carotene (β, β -carotene), zeaxanthin (β, β -carotene-3,3'-diol), astaxanthin (β, β -carotene-3,3'-diol-4,4'-diketo), lutein (β, ϵ -carotene-3,3'-diol) and alpha-carotene (β, ϵ -carotene).

Lycopene is a red carotenoid and has utility as a food colorant. Lycopene is naturally synthesised from the precursor phytoene through a series of four separate

dehydrogenation steps by the removal of eight atoms of hydrogen. Lycopene is an intermediate in the biosynthesis of other carotenoids in some bacteria, fungi, and all green plants.

- 5 Beta-carotene is an orange carotenoid that is naturally produced from lycopene through the intermediate gamma-carotene (β,ψ -carotene) by two sequential cyclization reactions that produce beta rings at the termini. Beta-carotene is useful as a colorant for margarine, butter and cheese, and as a provitamin which has been suggested to have a role in cancer prevention. Current commercial methods for producing beta-carotene include
- 10 isolation from carrots, chemical synthesis and microbial production.

- Zeaxanthin is a yellow carotenoid that is naturally produced from beta-carotene through the intermediate beta-cryptoxanthin by hydrogenation reactions which add hydroxyl groups to the beta rings at both termini. Zeaxanthin is used as a colorant in the poultry
- 15 industry. Zeaxanthin can be synthesized chemically, however, current chemical synthesis reactions are inefficient and are not commercially competitive. Therefore, zeaxanthin is usually produced by extraction from corn grain, and corn gluten meal. However, all of these plant sources are characterized by low and inconsistent production levels.

- 20 Alpha-carotene is another yellow carotenoid that is naturally produced from lycopene through the intermediate δ -carotene (ϵ,ψ -carotene) by two sequential cyclization reactions at the termini that produces one terminus with an epsilon ring and the other terminus with a beta ring. Alpha-carotene is useful as a colorant and as a provitamin.

- 25 Carotenoids have a variety of commercial uses ranging from use as a pigment to color foods and cosmetics to uses by the pharmacological industry. Pharmacological uses include use as a control during manufacture to distinguish one drug product from another, as an active component of various medicinal compositions, and as a vitamin supplement for humans. Carotenoids are also used as a dietary supplement in animal and poultry
- 30 feedstuffs. Carotenoids have even been used as a photoconductor in recording-media film.

In humans and animals carotenoids have diverse biological functions, and despite the similarity in structure, have different roles. Certain carotenoids are precursors to vitamin A

which can be converted to vitamin A by the body, examples are beta-carotene, alpha-carotene, and alpha-cryptoxanthin.

Aside from a role as a precursor to vitamin A, carotenoids are effective quenchers of
5 oxygen free radicals, with lycopene exhibiting the highest quenching activity. Carotenoids function as chain-breaking antioxidants and therefore protect the body from damage by free radicals. Free radicals have been implemented in a wide range of human ailments such as onset of pre-mature aging, cancer, atherosclerosis, cataracts, and an array of degenerative diseases. Carotenoids have also been shown to enhance the immune
10 system and to protect the skin from UV damage.

At present only a few plants are widely used to produce carotenoids. However, production of carotenoids from plants is expensive because of the low yields and variability of production. Recombinant DNA technology is a means for increasing the productive
15 capacity of carotenoid biosynthesis in plants.

In U.S. Patent No. 5,429,939 to Misawa *et al* DNA segments from *Erwinia uredovora* encoding bacterial enzymes geranylgeranyl pyrophosphate synthase, zeaxanthin glycosylase, lycopene cyclase, lycopene synthase, phytoene synthase, and beta-carotene
20 hydroxylase are disclosed. The abovementioned U.S. Patent provides a process for producing a carotenoid or a precursor compound in a host but the invention does not provide a means for controlling the ratio of specific carotenoids in a plant.

In U.S. Patent No. 5,530,188 to Ausich *et al* DNA segments encoding *Erwinia herbicola*
25 enzymes geranylgeranyl pyrophosphate, phytoene synthase, phytoene dehydrogenase-4H, and lycopene cyclase are disclosed. The abovementioned patent provides a means for producing beta-carotene in a plant containing the DNA segment encoding lycopene cyclase. However, the U.S. Patent does not provide a means for controlling the ratio of specific carotenoids in a plant thereby producing plants that produce other valuable
30 carotenoids.

In U.S. Patent No. 5,618,988 to Hauptmann *et al*, recombinant DNA technology was used to enhance carotenoid accumulation in the storage organs of genetically engineered plants by introducing into the plant a vector comprising a chimeric polypeptide consisting
35 of the bacterial gene encoding phytoene synthase conjugated to a plastid transit peptide.

The phytoene synthase was derived from the bacterium *Erwinia herbicola*. While the abovementioned U.S. Patent provides a means for increasing production of phytoene which then serves as a precursor to pigmented carotenoids, the patent does not provide a means for controlling the ratio of specific carotenoids in a plant thereby producing plants
5 that produce specific valuable carotenoids.

In U.S. Patent No. 5,684,238 to Ausich *et al* DNA segments from *Erwinia herbicola* encoding enzymes geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, beta-carotene hydroxylase, and
10 zeaxanthin glycosylase are disclosed. The abovementioned patent provides a means for producing zeaxanthin or glycosylated zeaxanthin in a culture containing a precursor and a host containing one or more said DNA segments or a transformed plant containing said beta-carotene hydroxylase. However, the U.S. Patent does not provide a means for controlling the ratio of other carotenoids in a plant thereby producing plants that produce
15 other valuable carotenoids.

In U.S. Patent No. 5,744,341 to Cunningham, Jr. *et al* DNA segments from *Arabidopsis thaliana* encoding the eucaryote enzymes epsilon-cyclase and beta-hydroxylase, and DNA segments from *Arabidopsis thaliana* and bacterium *Haematococcus pluvialis*
20 encoding the enzyme isopentyl pyrophosphate isomerase are disclosed. The U.S. Patent suggests uses for the disclosed DNA segments, however the patent does not provide a means for controlling the ratio of specific carotenoids in a plant species using DNA segments encoding various carotenoid biosynthesis enzymes from the same species thereby producing plants that produce other valuable carotenoids.

25

In U.S. Patent No. 5,750,865 to Bird *et al* DNA segments homologous to part or all of the clone pTOM from tomato is provided as a means to modify carotenoid biosynthesis in plants by promoting or inhibiting the synthesis of various carotenoids. The clone pTOM encodes an enzyme with a significant degree of homology to the crtB gene of
30 *Rhodobacter capsulatus* which encodes phytoene synthase. The abovementioned invention is used to promote or inhibit the carotenoid biosynthetic pathway, but the invention does not provide a means for controlling the ratio of specific carotenoids in a plant.

Although the above techniques have been successful in providing enhanced levels of certain carotenoids in bacterial hosts when the appropriate carotenoid precursor is provided to the host, it would be preferable to utilize a higher plant species wherein technical maintenance procedures would be minimized and yield of specific carotenoids could be optimized. While U.S. Patents to Hauptmann *et al* and Ausich *et al* disclose uses in higher plants, the carotenoid enzymes disclosed are of bacterial origin which are structurally distinct from the carotenoid enzymes of eucaryote origin. It is well known in the art that an enzyme from a bacterium can be functionally similar to an enzyme from a eucaryote, however, the enzymes are rarely structurally related and in many cases the enzymes can possess different secondary functions that in the heterologous host can be undesirable. While U.S. Patents to Bird *et al* and Cunningham *et al* disclose several DNA segments encoding carotenoid biosynthesis enzymes, the proposed uses for said DNA segments are in heterologous hosts which in certain cases may result in undesirable side effects.

15

Therefore, there still remains a need for isolation of DNA sequences encoding other carotenoid biosynthetic enzymes from other higher plants. There also remains a need to manipulate the carotenoid biosynthetic pathway in plants to enhance production of specific carotenoid compounds. Finally, there remains a need for transformed plant species, wherein each variety of transformed plant species comprises a combination of DNA sequences derived from a plant which when in the transformed plant species affects the accumulation of specific carotenoid compounds.

25 SUMMARY OF THE INVENTION

The present invention provides a transgenic plant material containing an isolated DNA encoding a marigold enzyme having catalytic activity of beta-cyclase. The present invention also provides a transgenic plant material containing an isolated DNA encoding a marigold enzyme having catalytic activity of beta-hydroxylase. The present invention further provides a transgenic plant material containing an isolated DNA encoding a marigold enzyme having catalytic activity of epsilon-cyclase, further still, and a transgenic plant material containing an isolated DNA encoding a marigold IPP isomerase. The present invention further provides a transgenic plant material containing more than one isolated DNA encoding a marigold enzyme having catalytic activity of an enzyme selected

from the group consisting of beta-cyclase, beta-hydroxylase, epsilon-cyclase, and isopentyl pyrophosphate (IPP) isomerase.

The present invention provides a transgenic plant material containing an isolated DNA
5 encoding a marigold enzyme having catalytic activity of beta-cyclase which produces an RNA antisense to an mRNA encoding beta-cyclase. The present invention also provides a transgenic plant material containing an isolated DNA encoding a marigold enzyme having catalytic activity of beta-hydroxylase which produces an RNA antisense to an mRNA encoding beta-hydroxylase. The present invention further provides a transgenic plant
10 material containing an isolated DNA encoding a marigold enzyme having catalytic activity of epsilon-cyclase which produces an RNA antisense to an mRNA encoding epsilon-cyclase. The present invention further provides a transgenic plant material containing more than one isolated DNA encoding a marigold enzyme having catalytic activity of an enzyme selected from the group consisting of beta-cyclase, beta-hydroxylase, and
15 epsilon-cyclase wherein the RNA produced by the isolated DNA is antisense to an mRNA encoding an enzyme selected from the group consisting of beta-cyclase, beta-hydroxylase, and epsilon-cyclase.

The present invention further provides a transgenic plant material containing more than
20 one isolated DNA encoding a marigold enzyme having catalytic activity of an enzyme selected from the group consisting of beta-cyclase, beta-hydroxylase, epsilon-cyclase and epsilon-hydroxylase wherein the RNA produced by at least one of the isolated DNAs is antisense to an mRNA encoding an enzyme selected from the group consisting of beta-cyclase, beta-hydroxylase, and epsilon-cyclase.

25

Thus, the present invention provides genetically engineered marigold plants that over-produce a desired carotenoid pigment in the petal. The present invention further provides a method for transforming marigold plants with various combinations of isolated DNAs which encode at least one of the enzymes selected from the group consisting of beta-
30 cyclase, epsilon-cyclase, beta-hydroxylase, IPP isomerase and epsilon-hydroxylase. The present invention allows the use of marigolds, a plant with known agronomic traits to produce a range of carotenoids in amounts that previously were not economically produced by traditional agricultural methods.

In the present invention, an isolated DNA encoding one or more of the enzymes selected from the group consisting of beta-cyclase, epsilon-cyclase, and beta-hydroxylase is operably linked to a promoter in the antisense orientation. The isolated DNA is introduced into the plant to make a transgenic plant. The isolated DNA in the plant is transcribed into
5 an antisense RNA which is complementary to the mRNA transcribed from the corresponding carotenoid biosynthesis pathway gene in the plant's genome. The antisense RNA and the plant's mRNA form a double-stranded RNA duplex with the mRNA which inhibits translation of the mRNA, preventing synthesis of the enzyme. The isolated DNA can range in length from 50 nucleotides to the full length of the mRNA.

10

In another embodiment of the present invention an isolated DNA encoding one or more of the enzymes selected from the group consisting of beta-cyclase, epsilon-cyclase, beta-hydroxylase, and IPP isomerase is operably linked to a promoter in the sense orientation. The isolated DNA is introduced into the plant to make a transgenic plant. The isolated
15 DNA in the plant is transcribed into an mRNA which is additive to the mRNA that is concurrently transcribed from the corresponding carotenoid biosynthesis pathway gene in the plant's genome. Thus an excess of mRNA encoding the desired carotenoid synthesis enzyme is produced. The excess mRNA is translated into the wanted enzyme producing an excess of the enzyme. Since there is now an excess of this enzyme, the excess
20 enzyme out competes with other enzymes in the pathway for substrate. Thus, the carotenoid biosynthesis pathway is shifted towards the direction of those carotenoid products produced by the wanted enzyme.

In a third embodiment, a first isolated DNA encoding one or more of the enzymes selected
25 from the group consisting of beta-cyclase, epsilon-cyclase, beta-hydroxylase, and epsilon-hydroxylase is operably linked to a promoter in the antisense orientation and a second DNA encoding one or more enzymes from the group not selected for antisense expression or IPP isomerase is operably linked to a promoter in the sense orientation. The isolated DNA is introduced into the plant to make a transgenic plant. The first DNA in
30 the plant is transcribed into an antisense RNA which is complementary to the mRNA transcribed from the corresponding carotenoid biosynthesis pathway gene in the plant's genome. The second isolated DNA in the plant is transcribed into an mRNA which is additive to the mRNA transcribed from the corresponding carotenoid biosynthesis pathway gene in the plant's genome causing an excess of the enzyme to be produced. The

simultaneous inhibition of certain of these enzymes and overproduction of other of these enzymes causes the preferential accumulation of specific carotenoid products.

The preferred promoter to produce the anti-sense or the sense RNA is a promoter that
5 specifically operates in the petals of the plant. Thus the carotenoid accumulates in the flower of the plant.

Transgenic plants containing the marigold genes regulated by the preferred petal-specific promoter allows the greatest level of production of the selected carotenoids in the petal of
10 the transgenic plant to be achieved without affecting other tissues of the plant.

OBJECTS

15 It is an object of the present invention to provide isolated DNA sequences from marigold plants which encode enzymes involved in the carotenoid biosynthesis pathway. The isolated DNA sequences encode enzymes selected from the group consisting of beta-cyclase, epsilon-cyclase, beta-hydroxylase, and IPP isomerase. It is also an object to provide a petal specific promoter to produce RNA from the isolated DNA in the petal of the
20 plant.

Another object of the present invention is to provide a method for producing a carotenoid in a marigold plant selected from the group consisting of beta-carotene, alpha-carotene, zeaxanthin, lycopene, zeinoxanthin, beta-cryptoxanthin, and combination thereof using
25 the abovementioned isolated DNA sequences to produce RNA in the plant that are antisense to the mRNA concurrently produced by the plant. Therefore, a plant transformed with a vector that produces RNA antisense to epsilon-cyclase mRNA will cause the plant to preferentially accumulate zeaxanthin; a plant transformed with vectors that produce RNA antisense to epsilon-cyclase and beta-cyclase mRNAs will cause the
30 plant to preferentially accumulate lycopene; a plant transformed with vectors that produce RNA antisense to epsilon-hydroxylase and beta-hydroxylase mRNAs will cause the plant to preferentially accumulate alpha-carotene; and a plant transformed with vectors that produce RNA antisense to epsilon-cyclase and beta-hydroxylase mRNAs will cause the plant to preferentially accumulate beta-carotene.

Another object of the present invention is to produce transgenic marigold which overproduce specific carotenoid biosynthesis enzymes which then causes the preferential accumulation of specific carotenoids in the petal. To accomplish the objective, the isolated DNA sequences are operably linked to a promoter in the sense orientation to produce a
5 mRNA in the sense orientation. The present invention further provides for transformed marigold plants containing one or more of the isolated DNA sequences in the plant which causes an excess of each of the enzyme encoded by the isolated DNA to be made. The excess enzyme encoded by the isolated DNA affects the ratio of specific carotenoids in the transgenic plant, causing the over accumulation of specific carotenoids. The ca-
10 rotenoids to be overproduced are selected from the group consisting of beta-carotene, alpha-carotene, zeaxanthin, lycopene, zeinoxanthin, beta-cryptoxanthin, rubixanthin, and combination thereof.

Further still an object of the present invention is to provide transformed marigold plants
15 containing various combinations of the isolated DNA sequences wherein certain DNA sequences are operably linked to a promoter which produce RNA in the sense orientation and other DNA sequences are operably linked to a promoter which produce RNA in the antisense orientation. The invention can be used to overproduce a carotenoid selected from the group consisting of beta-carotene, alpha-carotene, zeaxanthin, lycopene,
20 zeinoxanthin, beta-cryptoxanthin, rubixanthin, and combination thereof.

These and other objects will become increasingly apparent by reference to the following description and the drawings.

25 DETAILED DESCRIPTION OF THE DRAWINGS

Figure 1 is a flow diagram showing a part of the carotenoid pathway in higher plants.

Figure 2 is a flow diagram showing the reactions catalyzed by beta-cyclase and epsilon-
30 cyclase.

Figure 3 is the DNA sequence for beta-cyclase (SEQ ID NO:1).

Figure 4 is the amino acid sequence for beta-cyclase (SEQ ID NO:2).

Figure 5 is the DNA sequence for epsilon-cyclase (SEQ ID NO:3).

Figure 6 is the amino acid sequence for epsilon-cyclase (SEQ ID NO:4).

5 Figure 7 is the DNA sequence for beta-hydroxylase (SEQ ID NO:5).

Figure 8 is the amino acid sequence for beta-hydroxylase (SEQ ID NO:6).

Figure 9 is the DNA sequence for isopentyl pyrophosphate (IPP) isomerase (SEQ ID
10 NO:7).

Figure 10 is the amino acid sequence for IPP isomerase (SEQ ID NO:8).

15 DETAILED DESCRIPTION OF THE INVENTION

To facilitate the detailed description of the present invention, it is helpful to set forth definitions of certain terms to be used hereinafter.

20 Amino acids are the structural units comprising a polypeptide.

Nucleic acids are the structural units comprising a DNA or RNA molecule.

Transcription means the formation of an RNA chain in accordance with the genetic
25 information contained in the DNA. When the genetic information encodes a structural gene, the RNA so formed is referred to as mRNA.

Translation means the process whereby genetic information in a mRNA molecule directs the order of specific amino acids during protein synthesis.

30

Expression means the combination of cellular processes, including transcription and translation undergone by a structural gene to produce a polypeptide.

Expression vector means a plasmid or phage comprising a DNA sequence operably linked to a promoter which in a cell causes transcription of the DNA into an RNA molecule. The RNA molecule can be translated into a polypeptide.

- 5 Operably linked means a DNA sequence linked to a promoter wherein the promoter causes the DNA sequence to be transcribed into an RNA molecule. The DNA sequence can comprise a structural gene, a portion of a structural gene, or a structural gene or portion thereof in the antisense orientation.
- 10 Promoter means a DNA sequence which causes transcription of DNA into a RNA molecule. For purposes herein, promoter is used to denote DNA sequences that permit transcription in a plant.

Recombinant DNA molecule means a hybrid DNA sequence comprising at least two
15 nucleotide sequences not normally found together in nature.

Structural gene means a DNA sequence that is transcribed into an mRNA which is then translated into a polypeptide.

- 20 Vector means a DNA molecule that is capable of replicating in a cell and to which another DNA sequence can be operably linked so as to bring about replication of the attached DNA sequence. Commonly used vectors are bacterial plasmids and bacteriophages.

Sense refers to the sequence of the DNA strand of a structural gene that is transcribed
25 into an mRNA molecule copy which is then translated into the polypeptide encoded by the structural gene.

Antisense refers to the sequence of the DNA strand that is complementary to the sequence of the sense strand and cannot be translated into the polypeptide encoded by
30 the structural gene. For purposes of the present invention, antisense refers to a DNA that is operably linked to a promoter in the reverse orientation such that when the DNA is transcribed, an antisense RNA molecule is produced that has a nucleotide sequence that is complementary to and capable of hybridizing to an mRNA produced from the same DNA sequence in the sense orientation.

Polypeptide means the sequence of amino acids that comprise a structural gene. The term protein is equivalent to the term polypeptide. Enzymes are polypeptides.

Transformation means the process of introducing DNA into an organism which changes
5 the genotype of the recipient organism in a stable manner. Transformation encompasses the introduction of the DNA by whatever means.

Transgenic plant means a plant which by the process of transformation is made to contain DNA sequences which are not normally present in the plant or DNA sequences which are
10 in addition to the sequences which are normally present in the plant.

Polyadenylation site is the nucleotide sequence which causes certain enzymes to cleave mRNA at a specific site and to add a sequence of adenylic acid residues to the 3' end of the mRNA.

15

Marigold flowers have been used by the food and feed industries as a source of carotenoid pigments. The object of the present invention is to genetically engineer marigold plants to over-produce in the petals a desired carotenoid pigment. Marigold petals normally contain 1 to 3% zeaxanthin and greater than 90% lutein. Marigold plants
20 that preferentially accumulate other carotenoids can be made according to the present invention. Marigold plants transformed with various combinations of isolated DNAs which encode at least one of the enzymes selected from the group consisting of beta-cyclase, epsilon-cyclase, beta-hydroxylase, and IPP isomerase. The transformed marigold plants are genetically engineered wherein certain genes of the carotenoid biosynthesis pathway
25 (Figure 1) are either over-expressed or suppressed to deviate the carotenoid synthesis pathway in the desired direction which thus causes accumulation of desired carotenoids.

The carotenoids are preferably accumulated in the marigold flowers by using petal specific promoters operably linked to the abovementioned isolated DNAs. The petal-
30 specific promoter allows the modification of carotenoid biosynthesis to be relegated to the petals of the transgenic plant. This allows carotenoid production to be manipulated without affecting or harming other tissues of the plant. Standard technology can be used to isolate the accumulated carotenoids from the flowers of the transformed marigolds. The present invention allows the use of marigolds, a plant with known agronomic traits to produce a

range of carotenoids and in amounts that previously were not economically produced by traditional agricultural methods.

Carotenoids are the most widespread group of pigments found in virtually all
5 photosynthetic organisms and certain non-photosynthetic bacteria and fungi. In
photosynthetic organisms, carotenoids are an essential component of the photosynthetic
pathway. Glyceraldehyde-3-phosphate and pyruvate are used as substrates to produce
dimethylallyl pyrophosphat (DMAPP) by a series of reactions known as the alternative IPP
pathway. Many of the enzymes have yet to be described and cloned. DMAPP is
10 converted to IPP and then to geryanylgeranyl pyrophosphate (GGPP) through an
isomerization reaction catalyzed by IPP isomerase followed by a series of condensation
reactions by GGPP synthase. GGPP is dimerized by phytoene synthase to form
phytoene, the first C₄₀ carotenoid.

15 The part of the carotenoid biosynthesis pathway in higher plants that proceeds from
phytoene is shown in Figure 1. Phytoene is converted to the first pigment carotenoid,
lycopene, through a series of dehydrogenation reactions catalyzed by one or more
desaturases. Lycopene can serve as a precursor for a variety of other pigmented
carotenoids.

20

Lycopene can be converted to beta-carotene through two sequential cyclization reactions
catalyzed by beta-cyclase. Beta-cyclase cyclizes the termini of lycopene to form beta-
rings. The reactions catalyzed by beta-cyclase or epsilon-cyclase are shown in Figure 2.

25 Beta-carotene can then be converted to zeaxanthin by two sequential hydroxylation
reactions catalyzed by beta-hydroxylase which adds hydroxyl groups to the number 3
carbons of each beta-ring.

Lycopene can also be converted to alpha-carotene through two sequential cyclization
30 reactions, the first reaction is catalyzed by epsilon-cyclase which forms the intermediate
delta-carotene which has an epsilon-ring at one terminus and the second reaction,
catalyzed by beta-cyclase, cyclizes the other terminus to form a beta-ring. The reactions
are shown in Figure 1.

Alpha-carotene can be converted to alpha-cryptoxanthin in a reaction catalyzed by epsilon-hydroxylase which adds a hydroxyl group to the number three carbon of the epsilon-ring. A second hydroxylation reaction catalyzed by beta-hydroxylase converts alpha-cryptoxanthin to lutein by adding a hydroxyl group to the number three carbon of the beta-ring (Figure 1).

In addition to converting lycopene to beta-carotene, beta-cyclase can convert neurosporene to beta-zeacarotene which is then converted by a desaturase to gamma-carotene. Gamma-carotene can then be converted to beta-carotene by beta-cyclase or alpha-carotene by epsilon-cyclase. Neurosporene can also serve as a substrate for epsilon-cyclase which converts it into alpha-zeacarotene which is then converted to delta-carotene by a desaturase. Beta-cyclase can further convert delta-carotene to alpha-carotene.

Beta-hydroxylase can also convert alpha-carotene to zeinoxanthin which can then be converted to lutein in a reaction catalyzed by epsilon-hydroxylase.

The complexity of the pathway and the diversity of products formed in the reactions catalyzed by beta-cyclase, epsilon-cyclase, beta-hydroxylase, and epsilon-hydroxylase indicates that the pathway can be engineered to produce specific carotenoid products by altering expression of any one or several of the abovementioned enzymes.

Thus, the object of the present invention is to produce genetically engineered marigold plants which preferentially overproduce a desired carotenoid pigment in the petal. The present invention provides transgenic marigold plants which contain at least one of the isolated DNAs encoding the carotenoid biosynthesis gene selected from the group consisting of beta-cyclase, epsilon-cyclase, beta-hydroxylase, IPP isomerase, epsilon-hydroxylase, and combinations thereof to produce a transgenic marigold which preferentially accumulates in the petal a specific carotenoid biosynthesis pigment. The present invention provides isolated DNAs encoding beta-cyclase, epsilon-cyclase, beta-hydroxylase, and IPP isomerase from the marigold plant. The present invention also provides a method for transforming marigold plants with the isolated DNAs which encode at least one of the enzymes selected from the group consisting of beta-cyclase, epsilon-cyclase, beta-hydroxylase, IPP isomerase, epsilon-hydroxylase and combinations thereof

to produce a marigold plant which preferentially accumulates a specific carotenoid pigment in the petal.

Thus, the present invention provides an isolated DNA encoding beta-cyclase (Figure 3) wherein the isolated DNA has a sequence essentially the same as the sequence in SEQ ID NO.:1 wherein the sequence between positions 304 to 1836 encodes an enzyme having an amino acid sequence (Figure 4) essentially the same as the amino acid sequence in SEQ ID NO.:2. The isolated DNA of marigold encoding beta-cyclase was cloned in the plasmid pBSII KS+ (Stratagene, La Jolla, CA) which was deposited under the terms of the Budapest Treaty at the American Type Culture Collection (ATCC), 10801 University Blvd. Manassas, VA 20110-2209, USA on 28 July 1999 as ATCC PTA-447.

The present invention also provides an isolated DNA sequence encoding beta-hydroxylase wherein the isolated DNA has a sequence (Figure 7) essentially the same as the sequence in SEQ ID NO.:3 wherein the sequence between positions 51 to 923 encodes an enzyme having an amino acid sequence (Figure 8) essentially the same as the amino acid sequence in SEQ ID NO.:4. The isolated DNA of marigold encoding beta-hydroxylase was cloned in the plasmid pBSII KS+ which was deposited under the terms of the Budapest Treaty at the ATCC on 28 July as ATCC PTA-445.

The present invention further provides an isolated DNA sequence encoding epsilon-cyclase wherein the isolated DNA has a sequence (Figure 5) essentially the same as the sequence in SEQ ID NO.:5 wherein the sequence between positions 141 to 1688 encodes an enzyme having an amino acid sequence (Figure 6) essentially the same as the amino acid sequence in SEQ ID NO.:6. The isolated DNA of marigold encoding epsilon-cyclase was cloned in the plasmid pBSII KS+ which was deposited under the terms of the Budapest Treaty at the ATCC on 28 July as ATCC PTA-446.

The present invention further provides an isolated DNA sequence encoding IPP isomerase wherein the isolated DNA has a sequence (Figure 9) essentially the same as the sequence in SEQ ID NO.:7 wherein the sequence between positions 101 to 796 encodes an enzyme having an amino acid sequence (Figure 10) essentially the same as the amino acid sequence in SEQ ID NO.:8. The isolated DNA of marigold encoding IPP isomerase was cloned in the plasmid pBSII KS+ which was deposited under the terms of the Budapest Treaty at the ATCC on 28 July as ATCC PTA-448.

- In a first embodiment of the present invention, a transgenic plant material is provided containing at least one isolated DNA encoding a marigold enzyme selected from the group consisting of beta-cyclase, beta-hydroxylase, and epsilon-cyclase wherein the isolated DNA is operably linked to a RNA promoter which in the plant produces an RNA that is antisense to the mRNA encoding the corresponding enzyme which is concurrently being produced by the plant. The isolated DNA, operably linked to a promoter to produce the antisense RNA, is selected from the group consisting of SEQ ID NO.:1, preferably the sequence between positions 1 to 1836, SEQ ID NO.:3, preferably the sequence between positions 1 to 923, and SEQ ID NO.:5, preferably the sequence between positions 1 to 1688. The isolated DNA can range from 50 nucleotides to a length which corresponds to the length of the mRNA. In the preferred embodiment, the isolated DNA is operably linked to a promoter which is specific for transcription in the petal.
- 15 The present invention thus provides a method for producing a plant that preferentially accumulates either zeaxanthin, lycopene, alpha-carotene, beta-carotene, zeinoxanthin, or alpha-cryptoxanthin. The method comprises producing a transformed plant that contains a sequence selected from the group consisting of SEQ ID NO.:1, preferably the sequence between positions 1 to 1836, SEQ ID NO.3, preferably the sequence between positions 1 to 923, SEQ ID NO.:5, preferably the sequence between positions 1 to 1688 and combinations thereof, wherein the sequence is operably linked to a RNA promoter in the orientation which will produce an antisense RNA. The transformed plant produces the antisense RNA which inhibits the complementary mRNA (or pre-mRNA) produced by the plant that encodes the targeted carotenoid biosynthesis enzyme by forming a double-stranded RNA complex with the mRNA. The double-stranded complex is preferentially degraded by enzymes in the plant which are specific for double-stranded RNA thereby reducing the amount of the targeted mRNA. Since the concentration of mRNA encoding the targeted enzyme is reduced or eliminated, the quantity of the targeted enzyme product is reduced or eliminated which causes the preferential accumulation of those carotenoids that are substrates for the enzyme that is targeted.

Thus, in the method of the present invention for producing a plant that preferentially accumulates zeaxanthin, the isolated DNA encoding epsilon-cyclase is operably linked to a promoter in the orientation that in the transgenic plant is transcribed into an antisense RNA. The antisense RNA binds the mRNA that encodes epsilon-cyclase which prevents

synthesis of the epsilon-cyclase enzyme. The inhibition of epsilon-cyclase synthesis causes a decrease in epsilon-cyclase in the plant which then causes the transformed plant to preferentially accumulate the carotenoid zeaxanthin.

- 5 In the method for producing a plant that preferentially accumulates lycopene, the transgenic plant contains the isolated DNA encoding epsilon-cyclase and the isolated DNA encoding beta-cyclase, operably linked to a promoter in the orientation which produces antisense RNA. The antisense RNAs bind the mRNAs encoding epsilon-cyclase and beta-cyclase, respectively, thereby preventing synthesis of the epsilon-cyclase and
- 10 beta-cyclase enzymes. The decrease of the beta-cyclase and epsilon-cyclase enzymes causes the transformed plant to preferentially accumulate lycopene.

- In the method for producing a plant that preferentially accumulates alpha-carotene, the transgenic plant contains the isolated DNA encoding epsilon-hydroxylase and the isolated
- 15 DNA encoding beta-hydroxylase, operably linked to an promoter in the orientation which produces antisense RNA. The antisense RNAs bind to the complementary RNAs encoding epsilon-hydroxylase and beta-hydroxylase, respectively, preventing synthesis of epsilon-hydroxylase and beta-hydroxylase. The decrease of epsilon-hydroxylase and beta-hydroxylase causes the transformed plant to preferentially accumulate alpha-
- 20 carotene.

- In the method for producing a plant that preferentially accumulates beta-carotene, the transgenic plant contains the isolated DNA encoding epsilon-cyclase and the isolated DNA encoding beta-hydroxylase, operably linked to a promoter in the orientation which
- 25 produces antisense RNA. The antisense RNAs bind their respective complementary mRNA which inhibits synthesis of the enzymes for beta-hydroxylase and epsilon-cyclase. The decrease of these enzymes causes the transformed plant to preferentially accumulate beta-carotene.

- 30 In the method for producing a plant that preferentially accumulates zeinoxanthin, the transgenic plant contains the isolated DNA encoding epsilon-hydroxylase, operably linked to a promoter in the orientation which produces antisense RNA. The antisense RNA binds the mRNA that encodes epsilon-hydroxylase which prevents synthesis of the epsilon-hydroxylase enzyme. The inhibition of epsilon-hydroxylase synthesis causes a decrease

of the epsilon-hydroxylase in the plant which then causes the transformed plant to preferentially accumulate the carotenoid zeinoxanthin.

In the method for producing a plant that preferentially accumulates alpha-cryptoxanthin,
5 the transgenic plant contains the isolated DNA encoding beta-hydroxylase, operably linked to a promoter in the orientation which produces antisense RNA. The antisense RNA binds the mRNA that encodes beta-hydroxylase which prevents synthesis of the beta-hydroxylase enzyme. The inhibition of synthesis causes a decrease of the enzyme in the plant which then causes the transformed plant to preferentially accumulate the
10 carotenoid alpha-cryptoxanthin.

In the aforementioned embodiments, the promoter that is operably linked to the isolated DNA to make the antisense RNA is a promoter that causes the transcription of the RNA from the isolated DNA to occur specifically in the petal of the marigold. An example of an
15 RNA promoter that is specific for transcription in the petal is the Adonis ketolase promoter.

The present invention provides a transgenic plant material containing one or more isolated DNAs encoding marigold enzymes selected from the group consisting of beta-cyclase, beta-hydroxylase, epsilon-hydroxylase, IPP isomerase and epsilon-cyclase
20 wherein the beta-cyclase is encoded by the nucleotide sequence essentially homologous to the sequence between positions 1 to 1836 in SEQ ID NO.:1, the beta-hydroxylase is encoded by the nucleotide sequence essentially homologous to the sequence between positions 1 to 923 in SEQ ID NO.:3, the epsilon-cyclase is encoded by the nucleotide sequence essentially homologous to the sequence between positions 1 to 1688 in SEQ ID
25 NO.: 5, the epsilon-hydroxylase and the IPP isomerase is encoded by the nucleotide sequence essentially homologous to the sequence between positions 1 to 796 in SEQ ID NO.:7. The isolated DNA is operably linked to a promoter which in the host produces a functional mRNA that encodes the enzyme. In the preferred embodiment, the isolated DNA is operably linked to a promoter that is specific for transcription in the petal.

30

In another embodiment, the present invention provides a transgenic plant material containing combinations of isolated DNAs encoding marigold enzymes selected from the group consisting of beta-cyclase, beta-hydroxylase, epsilon-hydroxylase, IPP isomerase and epsilon-cyclase wherein a first isolated DNA sequence is operably linked to a
35 promoter to produce antisense RNA and a second isolated DNA sequence is operatively

linked to a promoter to produce an RNA that produces a functional enzyme. To produce the functional enzyme, the promoter is operably linked in the sense orientation to either beta-cyclase encoded by the nucleotide sequence essentially homologous to the sequence between positions 1 to 1836 in SEQ ID NO.:1, the beta-hydroxylase encoded by the nucleotide sequence essentially homologous to the sequence between positions 1 to 923 in SEQ ID NO.:3, the IPP isomerase encoded by the nucleotide sequence essentially homologous to the sequence between positions 1 to 796 SEQ ID NO.:7, the epsilon-hydroxylase or the epsilon-cyclase encoded by the nucleotide sequence essentially homologous to the sequence between positions 1 to 1688 in SEQ ID NO.:5. To produce the antisense RNA, the isolated DNA is operably linked to the promoter in the antisense orientation and the length of the isolated DNA can range from 50 nucleotides to a length which corresponds to the full length of the mRNA. In the preferred embodiment, the isolated DNA is operably linked to a promoter that is specific for transcription in the petal.

Thus, the present invention provides a means for manipulating the carotenoid pathway in a plant to overproduce specific carotenoids and repress production of other carotenoids. For example, the present invention provides a means for inhibiting synthesis of epsilon-cyclase by introducing into the plant, DNA encoding RNA antisense to the epsilon-cyclase mRNA produced by the plant. Inhibition of epsilon-cyclase by the hybridization of the antisense RNA to the mRNA prevents synthesis of epsilon-cyclase which then reduces or prevents the conversion of neurosporene to alpha-zeacarotene, lycopene to delta-carotene, and gamma-carotene to alpha-carotene. Therefore, the carotenoid biosynthetic pathway will preferentially proceed towards the production of zeaxanthin. Inhibiting beta-hydroxylase in the same manner will prevent conversion of beta-carotene to zeaxanthin and zeinoxanthin to lutein, thereby causing the accumulation of beta-carotene and zeinoxanthin.

In a second example according to the present invention, inhibition of the synthesis of the beta-cyclase and epsilon-cyclase enzymes is accomplished by introducing into the plant DNA encoding RNAs antisense to the beta-cyclase and epsilon-cyclase mRNAs produced by the plant. The antisense RNAs bind to their respective complementary mRNAs which inhibits translation of their respective mRNAs, thereby inhibiting synthesis of the beta-cyclase and epsilon-cyclase enzymes. The inhibition of the synthesis of the beta-cyclase and epsilon-cyclase enzymes reduces or eliminates conversion of neurosporene to beta-

zeacarotene and lycopene to beta-carotene and delta-carotene. Therefore, the primary product of the carotenoid in a pathway is lycopene.

In a third example according to the present invention, inhibition of synthesis of the beta-
5 hydroxylase and epsilon-hydroxylase enzymes will cause the preferential accumulation of alpha-carotene. It also follows from this example that inhibition of beta-hydroxylase according to the present invention prevents alpha-cryptoxanthin from being converted to lutein, thereby causing accumulation of alpha-cryptoxanthin, and that inhibition of only
10 epsilon-hydroxylase prevents zeinoxanthin from being converted to lutein, thereby causing accumulation of zeinoxanthin.

The present invention also provides for manipulation of the carotenoid biosynthesis pathway wherein any one of the abovementioned enzymes is overproduced in the plant. For example, overproduction of beta-cyclase according to the present invention will
15 produce an excess of beta-cyclase which will more effectively compete with epsilon-cyclase for neurosporene and lycopene substrates thereby causing the carotenoid biosynthesis pathway to preferentially increase production of beta-carotene and zeaxanthin, and decrease production of alpha-carotene and its derivatives. Conversely, overproduction of epsilon-cyclase will cause the carotenoid biosynthesis pathway to shift
20 towards production of alpha-carotene and its derivatives. Therefore, the present invention encompasses manipulation of the carotenoid biosynthesis pathway by providing to the plant, an isolated DNA containing at least one of the enzymes selected from the group consisting of beta-cyclase, beta-hydroxylase, epsilon-cyclase and epsilon-hydroxylase which when transcribed into mRNA and translated in the plant, provides an additional
25 amount of the carotenoid biosynthesis enzymes selected to be overproduced.

The genes encoding beta-cyclase, epsilon cyclase and beta-hydroxylase were isolated from marigold and cloned into a bacterial plasmid. The DNA sequence for beta-cyclase is shown in Figure 3. The gene encoding the beta-cyclase is 1533 bp and corresponds to
30 nucleotide position 304 to 1836. The amino acid sequence for beta-cyclase is shown in Figure 4. The DNA sequence for epsilon-cyclase is shown in Figure 5. The gene encoding the epsilon-cyclase is 1548 bp and corresponds to nucleotide position 141 to 1688. The amino acid sequence for epsilon-cyclase is shown in Figure 6. The DNA sequence for beta-hydroxylase is shown in Figure 7. The gene encoding the beta-hydroxylase is 873 bp
35 and corresponds to nucleotide position 51 to 923. The amino acid sequence for beta-

cyclase is shown in Figure 8. The DNA sequence for IPP isomerase is shown in Figure 9. The gene encoding for IPP isomerase is 796 bp and corresponds to nucleotide positions 101 to 796. The amino acid sequence for IPP isomerase is shown in Figure 10. The petal specific promoter was isolated from *Adonis vernalis* and is the promoter regulating the
5 ketolase gene. The marigold genes encoding geranylgeranyl pyrophosphate synthase and zeta-carotene desaturase have been cloned and sequenced.

Construction of clones containing the carotenoid biosynthesis DNA operably linked to a promoter can be accomplished using techniques well known in the art (for example
10 Sambrook et al (1989)). Suitable vectors for eukaryote expression in plants are described in Frey et al (1995), and Misawa et al (1994), which are incorporated herein by reference.

Transgenic plants are constructed which contain the DNA sequences comprising the present invention. The incorporation of these sequences into the plant allows the
15 carotenoid biosynthetic pathway to be manipulated to produce specific carotenoids. The manipulation can be by antisense inhibition, overproduction of selected carotenoid biosynthesis enzymes, or a combination thereof.

There are many methods known in the art for transforming a plant cell. Common methods
20 include transformation with T-DNA containing the DNA of interest and using *A. tumefaciens* as the means for transformation or with Ti or Ri plasmids using the bacterium *A. rhizogenes* as the means for transformation. A suitable plasmid for transformations is the pART27/7 plasmid vector isolated from *Agrobacterium tumefaciens*. Other methods for transforming a plant cell include cell fusion, electroporation, biolistic or conventional
25 injection.

Agrobacterium related methods require special plasmid vectors such as intermediate or binary vectors. Intermediate vectors require integration into Ti or Ri plasmids by homologous recombination into the region containing the T-DNA. The intermediate vector
30 is transferred into the *Agrobacterium* by means of conjugation in the presence of a helper plasmid. The transformed *Agrobacterium* is then used to transform the cell. The preferred method for transforming *Agrobacterium* is using plasmids of the binary type. Binary vectors replicate both in *Escherichia coli* and *Agrobacterium*. Therefore, these vectors containing the desired DNA can be constructed using conventional molecular biology
35 techniques and the recombinant plasmid directly transferred to *Agrobacterium*. Binary

vectors usually contain a marker gene and a polylinker for inserting the desired DNA flanked by the left and right T-DNA border regions. Both the intermediate and binary vectors contain the *vir* region which is necessary for transfer of the T-DNA into the plant cell.

5

Transformation of plant cells with transformed *Agrobacterium* is by co-cultivation of the cells with the transformed *Agrobacterium* which results in transfer of the T-DNA containing the desired DNA into the plant cell. Sources for plant cells are explants which can include but is not limited to sections of leaves, stems, roots, segments of petioles, 10 flowers and flower parts, and cotyledon tissue. Whole plants are regenerated from the infected plant material or from protoplasts or suspension-cultivated cells in a suitable medium which can contain antibiotics or biocides (e.g., kanamycin, bleomycin, hygromycin, chloramphenicol) for selection of the transformed plant cells. The ability and efficiency of regenerating a transformed or transgenic plant using transformed isolated 15 cells or explants is dependent on the species of plant and the type of transformed cell. Transformation of marigold tissue can be achieved according to the *Agrobacterium*-mediated method for transforming plants disclosed in U.S. Patent Nos. 5,684,238 to Ausich *et al* and 5,618,988 to Hauptmann *et al* which are herein incorporated by reference.

20

Non-*Agrobacterium* mediated transformation such as electroporation, injection, cell fusion, or particle bombardment do not require special plasmids and can therefore use standard plasmids such as the pUC derivatives and conventional cloning techniques. For example, to make the transgenic marigold plants of the present invention using the Biolistic 25 bombardment method, marigold tissue is transformed using the Biolistic method described in U.S. Patent No. 5,767,368 to Zhong *et al* which is herein incorporated by reference. Further examples of the Biolistic bombardment method are disclosed U. S. Patent No. 5,736,369 to Bowen *et al* which is herein incorporated by reference.

30 Expression of cloned DNAs such as the isolated DNAs of the present invention in the plant cell requires the isolated DNA to be operably linked to a promoter. The preferred promoter is the petal specific promoter from the ketolase gene of *Adonis vernalis* (pheasant's eye). Examples of other promoters which are useful are viral promoters such as the cauliflower mosaic virus 35S promoter, heat shock protein promoters such as the 35 HSP70 promoter, light induced promoters such as the ST-Ls1 or the rubisco small subunit

promoter, stress response promoters such as the PR promoter, the *Agrobacterium tumefaciens nos* promoter, and various organ, root, tuber, leaf, and other flower specific promoters. Examples of other promoters contemplated are differentially regulated promoters which are promoters that operate in only certain plant tissues, under certain
5 environmental conditions or at a particular developmental stages of the plant. The CRB promoter isolated from the CRB gene of the 12S seed protein of *Arabidopsis thaliana* which targets expression to the seed is one such differentially regulated promoter. The DRE promoter element that is inducible under stress is an example of a plant promoter that responds to environmental conditions (Yamaguchi-Shinozaki et al, 1994). The
10 isolated DNA also requires being operably linked to a transcription termination signal. The termination signal can be the sequence naturally associated with the isolated DNA or can be a sequence operably linked to the 3' end of the isolated DNA. An example of such a sequence is the transcription termination signal of the octopine synthase gene.

15 In the embodiments of the invention wherein antisense RNA production is desired, the transcription of the isolated DNAs in the plant cell produces an RNA that is antisense to the mRNA or pre-mRNA of the gene product targeted for inhibition. James (1991) has reviewed antisense RNA and its use in gene inhibition therapy. Other reviews of antisense technology specifically directed to transgenic plants are by Senior (1998) and Nellen *et al.*
20 (1996). Generally, the inhibition is affected in the cell nucleus by the formation of a double-stranded RNA consisting of one molecule of antisense RNA and one molecule of the mRNA forming a double helix molecule. The double helix molecule is preferentially degraded in the nucleus by enzymes that specifically degrade double-stranded RNA molecules. In this manner, the pool of mRNA available for translation is reduced or
25 eliminated which in turns reduces the pool of enzyme encoded by the mRNA. The length of the antisense RNA that is effective for inhibition is between 50 nucleotides and a size which corresponds to the full length of the mRNA it is complementary with. The degree of inhibition affected by the present invention is at least 70% such as at least 80% including 90% preferably at least 98%, depending on the length of the antisense RNA and the
30 particular region of the mRNA it is directed to when the antisense RNA is shorter than the mRNA. Thus, the present invention provides a method for substantially inhibiting a particular enzyme by using an RNA that is antisense to the enzymes mRNA.

The present invention describes transgenic marigold plants wherein the carotenoid
35 biosynthesis pathway is manipulated to produce specific carotenoids by transforming

marigold tissue with various combinations of one or more isolated DNAs containing beta-cyclase, epsilon-cyclase, beta-hydroxylase, IPP isomerase or epsilon-hydroxylase in either antisense or sense orientation. However, manipulation of the carotenoid pathway according to the present invention can include other enzymes that are involved in the biosynthesis of carotenoids. These enzymes can be of marigold origin or from other organisms. Examples of such genes are the 1-deoxy-D-xylulose 5-phosphate synthase (DXP synthase) from *E. coli* (GenBank Accession No. U82664), the marigold homolog to the DXP synthase which produces a deep red when in the presence of lycopene, and the *Arabidopsis thaliana* homolog to DXP synthase (Cla 1 gene - GenBank Accession No. U27099). Thus, the present invention is not limited to the specific genes mentioned herein but also includes other genes encoding enzymes that are involved in carotenoid biosynthesis.

The following examples are intended to promote a further understanding of the present invention.

EXAMPLE 1

HPLC characterization of selected marigold lines including known color variants was performed to identify marigold color variants that had mutations in the carotenoid biosynthetic pathway. These mutations were expected to accumulate intermediates such as beta or alpha carotene or mono-hydroxy derivatives.

In normal orange marigold lines between 90 and 98% of their total carotenoid content is lutein. The vast majority of the lutein is esterified to fatty acids. HPLC analysis was performed on all commercially available marigold color variants such as the dark orange, red fringed, yellow, cream, and white variants among others. All the commercially available variants were identified as quantitative mutants, that is these variants accumulated less of each intermediate in the same proportion. In other words, none of the variants accumulated any intermediate at appreciable levels. Therefore, marigold variants that have useful carotenoid mutations that cause accumulation of carotenoid biosynthetic pathway intermediates appeared to be distant.

EXAMPLE 2

A cDNA library was constructed to screen for and isolate cDNAs encoding enzymes involved in the carotenoid biosynthetic pathway. To facilitate construction of the cDNA library, the mRNA levels for carotenoid biosynthetic steps during marigold flower development was analyzed to identify the appropriate stage of development to prepare the cDNA library. The cDNAs targeted were cDNAs encoding beta-cyclase, epsilon-cyclase, beta-hydroxylase, IPP isomerase and epsilon hydroxylase. It was also discovered that the corresponding cDNAs encoding beta-cyclase, epsilon-cyclase, and beta-hydroxylase from *Arabidopsis thaliana* hybridized to the corresponding marigold genes. This discovery enabled expression of the abovementioned carotenoid pathway mRNAs be directly evaluated during floral development.

Based on the analysis of mRNA levels, three of six arbitrary marigold floral development stages were selected for sources of RNA for library construction. A cDNA library containing more than 10^7 independent cDNAs was constructed and screened for cDNAs encoding beta-cyclase, epsilon-cyclase, beta-hydroxylase, and epsilon-hydroxylase. Briefly, poly(A+) RNA was isolated from developing marigold flowers and made into cDNA using art known methods. A cDNA library was made by Stratagene (La Jolla, California) using the Stratagene Lambda ZAP Cloning System. The library was non-directional in the vector and consisted of more than 10^7 independent clones. Various screening procedures were used including heterologous screening using relevant *Arabidopsis* genes, functional screening based on colour complementation and novel methods based on accelerated growth at low temperature. Identification of clones containing carotenoid biosynthesis enzymes was as follows.

Marigold beta-cyclase was identified by colour complementation of a lycopene accumulating *E. Coli* strain. This method is described in U.S. Patent No. 5,744,341 to Cunningham, Jr. *et al* which is herein incorporated by reference. Approximately 360,000 colonies were screened. Of these colonies, 4 yellow colonies were picked, and DNA was extracted from two of the colonies and the DNA sequenced. Figure 3 shows the DNA sequence for the marigold beta-cyclase. The amino acid sequence for beta-cyclase was deduced from the DNA sequence and is shown in Figure 4.

Marigold epsilon-cyclase was identified by plaque hybridizations using as the probe the *Arabidopsis thaliana* epsilon-cyclase (GenBank Accession No: U50738). A DNA clone containing an epsilon cyclase from *Arabidopsis thaliana* that is suitable for use as a probe to screen the library is available from the ATCC as ATCC-98005. Approximately 280,000
5 plaques were screened and 9 plaques were purified. DNA was isolated from 2 of the plaques and the DNA was sequenced. The DNA sequence is shown in Figure 5. The amino acid sequence for epsilon-cyclase was deduced from the DNA sequence and is shown in Figure 6.

10 Marigold beta-hydroxylase was identified by plaque hybridizations using as the probe the *Arabidopsis* beta-hydroxylase. A DNA clone suitable for use as a probe to screen the library is available from the ATCC as ATCC-98003. Approximately 280,000 plaques were screened and 13 plaques were purified. DNA was isolated from 3 plaques and the DNA was sequenced. The DNA sequence is shown in Figure 7. The amino acid sequence for
15 beta-hydroxylase was deduced from the DNA sequence and is shown in Figure 8.

Marigold IPP isomerase was identified by using a cold screen method in which zeaxanthin expressing *E. coli* were transformed with the marigold cDNA library and grown at 18°C. Rapidly growing pigmented colonies which contained the IPP isomerase were

20 characterized. Five independent colonies were further shown to contain marigold IPP isomerases. Four of these clones were partially sequenced and one of these clones was fully sequenced. All of these clones were closely related but not identical. The DNA sequence is shown in Figure 9. The amino acid sequence for IPP isomerase was deduced from the DNA sequence and is shown in Figure 10.

25

Clones from a marigold cDNA library encoding geranylgeranyl pyrophosphate synthase and zeta-carotene desaturase have been identified by homology to their homologous genes in *Arabidopsis*. These genes have been isolated and sequenced.

30 EXAMPLE 3

Regeneration of marigold plants is a key element for successful generation of transgenic plants, however there is little information regarding tissue culture of marigold. Therefore, the objective of this example was to develop a method for the regeneration of marigold *in*

vitro. As part of our objective, several commercial and proprietary marigold genotypes were evaluated for germination and growth in culture.

Identification of marigold genotypes that regenerated best *in vitro* was performed by evaluating the number of adventitious shoots per experiment. All varieties of marigold plant tissue were evaluated.

Table 1: Summary of the morphological responses of marigold tissues to various hormonal concentrations and combinations.

MEDIA		TISSUE RESPONSE
BA (1.0 mg/l)	IAA (1.0 mg/l)	R-
	IAA (3.0 mg/l)	R-S-
	IAA (5.0 mg/l)	C+
BA (3.0 mg/l)	IAA (1.0 mg/l)	S++, R-, C+
	IAA (3.0 mg/l)	S++
	IAA (5.0 mg/l)	S+, C+
BA (5.0 mg/l)	IAA (1.0 mg/l)	S++, C+
	IAA (3.0 mg/l)	S+, C+
	IAA (5.0 mg/l)	S-, R+, C+
BA (1.0 mg/l)	IAA (0.5 mg/l)	S+, C++
BA (5.0 mg/l)	IAA (3.0 mg/l)	S++++, R+, C++

10 R= roots, S= shoots, C= callus

+++ = excellent development; ++ = very good development; + = good development; - = poor development

Regeneration potential of marigold was evaluated by monitoring the morphological response of marigold tissues to various hormonal concentrations and combinations. Regeneration was evaluated in three stages: shoot induction, shoot elongation, and rooting. The first stage, shoot induction, was performed as follows. The media was Murashige and Skoog (MS) medium containing various concentration of benzyladenine (BA) ranging from 1.0 mg/l to 5.0 mg/l. At each concentration of BA, various concentrations of IAA were added, ranging from 0.5 mg/l to 5.0 mg/l. Table 1 shows that MS media containing 5.0 mg/l BA and 3.0 mg/l IAA was the best medium for regenerating transformed marigold cultures.

Shoot buds, differentiated as above, are subcultured in the same media as above every two weeks for multiplication of shoots, as long as the regeneration from callus continues. Once shoots are visible from callus or original explants they are subcultured to MS media containing one tenth of the hormones used for shoot induction.

5

In the next stage, shoot elongation, shoot buds from above are subcultured on MS media without BA and IAA. In the final stage, rooting, the tissue from the shoot elongation stage is further subcultured in media without BA and IAA.

- 10 Table 2 summarizes the response of different marigold explants to media containing different hormones.

Table 2: Summary of the response of different marigold explants to media containing different hormones.

MARIGOLD VARIETY	MORPHOGENIC RESPONSE	STATUS
CLIMAX HYBRID TOREADOR (1)	S+, R+, C+, NGR+	
GOLDEN CLIMAX HYBRID	S++, R++, C+, NGR++	
XANTHOPHYLL SCARLETADE	S+, C++	
XANTHOPHYLL ORANGEADE	S+, C++	
XANTHOPHYLL DEEP ORANGE	S+, C++	
032-442 (5287)	S-, C++, NGR++	leaf, stem, cotyledon
032-439 (1273)	S++, R++, C++, NGR-	leaf, stem, cotyledon
36969	S++, R++, C++, NGR-	cotyledon
36898	S++, R++, C++, NGR-	cotyledon
032-440 (1274)	S+, R-, C++, NGR-	cotyledon

- 15 R= roots, S= shoots, C= callus, NGR= negative geotropic roots
 +++ = excellent development; ++ = very good development; + = good development; - = poor development

There were recurring problems with most genotypes which was manifested as browning of the tissue and growth of non-geotropic roots (growth of roots can be a problem during regeneration, because once the roots start to form, the growth of other plant structures decreases). However, in terms of regeneration of marigold plants from untransformed
5 tissue, many plants have been regenerated from different explants, tissues and genotypes according to the method shown herein.

EXAMPLE 4

10 Initial marigold tissue culture transformations were performed to determine the appropriate tissue for transformation with *Agrobacterium* and then plant regeneration from transformed tissue. The *Agrobacterium* that was used was *Agrobacterium* LB4404 containing in most cases the transformation vector pBI121 which contained the CaMV 35S promoter driving the beta-glucuronidase reporter gene (GUS) and the NPTII gene as
15 the selectable marker. The beta-glucuronidase cleaves the colorless substrate, X-glu, producing a product having a blue color.

Leaves were selected as the tissue from marigold for transformation because leaves are generally an easy regenerating tissue providing healthy plants back from leaves in culture
20 after approximately eight to six weeks. Large-scale transformations were initiated in earnest using six Pan American marigold lines in case there were cultivar variations as in tomato which would affect transformation. Over 5,000 independent leaf sections were individually transformed by *Agrobacterium*-mediated transformation and carried through regeneration attempts for approximately eight to twelve weeks, with weekly or bi-weekly
25 transfers for each transformation event. Despite the number of transformations not a single transformation event scored as transformed plantlets were ever identified even though transformed callus tissue that proliferated roots could be obtained. However, the transformed callus tissue was recalcitrant to plant regeneration.

30 It was observed that during these transformation attempts, many of the transformed tissues turned brown, would not show any response to hormones, and eventually died. Several alternative approaches were tried to transform marigold leaf tissue. Among them being using different tissues for transformation, and using other strains of *Agrobacteria* as the transforming agent. Because marigolds produce thiophenes which are natural
35 antibacterial compounds and may inhibit *Agrobacterium*-mediated transformation,

transformants were co-cultivated in the dark (light activates thiophenes), transformations were performed with low thiophene producing strains of marigolds, or transformants were co-cultivated in sulfate deficient media (to decrease thiophene production *in vitro*). None of these variations produced transformed plants. Therefore, the conclusion was that

5 despite the ability of marigold leaf tissue to regenerate better than any other plant tissue, marigold leaves were difficult to transform (less than 1% efficiency) and the tissue that was transformed could not be made to regenerate into plants.

Since marigold leaves were refractory to regeneration after transformation, other marigold

10 tissue was evaluated for regeneration and transformation. Marigold cotyledon tissue was tested for ability to be transformed. Cotyledon tissue was transformed with *Agrobacterium* LB4404. Transformed cotyledon tissue is capable of transformation several independent transformation events have produced transformed plants capable of growth in soil.

15 The protocol for marigold transformation that was developed using *Agrobacterium* is set forth below.

Induction and inoculation. Two weeks prior to the experiment, germinate seeds aseptically in MS media and agar plates. Two days prior to inoculation, cut off cotyledons

20 from seedlings and place them on MS media containing hormones as described in Example 3, and incubate under standard conditions. One to two days prior to inoculation, streak *A. tumefaciens* onto a petri plate containing LB agar and grow for two days with appropriate antibiotics.

25 On day of inoculation, scrape the new growth bacteria from the culture plate and make a mixture using induction medium in MS media. Shake the mixture for 30 minutes before using. Using sterile forceps transfer all cotyledons to a plate and then add the bacteria mixture and vacuum infiltrate for 5 minutes. Then remove all explants from the bacteria mixture and place the bacteria coated explants back into the same media they had been

30 growing in for co-cultivation. The co-cultivation period allows the bacteria and plant material to remain in close proximity for 2 to 3 days. After the co-cultivation period, transformed plant tissue is selected by transferring all the explants to the same media containing antibiotics to kill the *Agrobacterium* and kanamycin or hygromycin to select for transformants. Transformants can also be selected for herbicide resistance, provided that

35 the transformed tissue is cotransformed with DNA encoding a herbicide resistance gene

and the selection is performed in media containing the herbicide. Regeneration is essentially as described in Example 3.

The following three transformation experiments were done using the 35S-GUS-HYG construct (similar to pBI121 except encoding resistance to the antibiotic hygromycin) in LB4404. The explant used was cotyledons from aseptically grown marigold seedlings for each genotype. Regeneration was essentially as described in Example 3. The results shown in Table 3, demonstrate that using the transformation conditions and regeneration conditions described herein, cotyledon tissue from marigolds can be transformed and regenerated into plants. This important discovery provides both the method and transgenic marigold of the present invention.

Table 3: Summary of transformation experiment using the 35S-GUS-HYG construct.

Variety	No. Explants inoculated	Transformants GUS positive	Total plantlet clones (after subculture)
032-439 (1273	320	6	48
36969	300	5	28
36969	350	4	25

The use of cotyledons allowed plantlets to be regenerated following inoculation with *Agrobacterium*. Even though the transformation efficiency of cotyledon was not much better than the efficiency for transforming leaves, the transformed cotyledon tissue is capable of being regenerated into plants. Currently, there are two transformed plants from transformed 032-439 in the soil. There is one transformed 36969 plant in the soil with several others ready for planting. Thus, the method developed herein will produce transgenic marigold plants from transformed cotyledon tissue.

EXAMPLE 5

As an alternative to *Agrobacterium* mediated transformation, each transformation of the nuclear genome of marigold is accomplished by transforming marigold tissue such as cotyledon tissue or shoot-tips with one of the three isolated DNAs. The DNAs are coprecipitated onto 1.0 μ m tungsten particles according to the method described by

U.S. Patent No. 5,320,961 to Zhong et al. Multiple marigold shoot-tip clumps are initiated from shoot tips of marigold seedlings and maintained in light for 4-week intervals on Murashige and Skoog (MS) medium containing 2 mg/ml benzyladenine (BA) and 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). Shoot tips and shoot clumps are physically
5 exposed by removal of the leaves, when necessary, and placed in a circular area having a diameter of approximately 1.5 cm prior to transformation. Alternatively, cotyledon tissue can be transformed by Biolistic bombardment.

Transformation consists of bombarding the shoot tips and clumps with the tungsten
10 particles coated with the DNA precipitate using a Biolistic particle acceleration device (PDS 1000/He, Bio-Rad, Hercules, CA USA) under a chamber pressure of 26 mm of Hg at distances of 1.5, 2.0 and 6.5 cm from the rupture disc to the macrocarrier to the stopping screen to the target, respectively, with a density of 150 µg/shot of the coated tungsten particles with 4 shots and 1,550 p.s.i. acceleration pressure.

15 Afterwards, the bombarded tissue is cultured on MS medium containing 2 mg/ml BA and 0.5 mg/L 2,4-D for 6 to 8 weeks. This important step is necessary to reduce the degree of chimerism in the transformed tissue. Afterwards, the green clumps are selected, divided and subcultured in the above medium. Then, those plantlets that have normal root
20 development are transferred to pots and acclimated to soil conditions before being transferred to greenhouses.

Production of specific carotenoid compounds is determined using methods described in Example 1. In addition, a selection method such as antibiotic resistance (Example 5) or
25 herbicide resistance can be incorporated into this method by co-transforming the plant tissue an isolated DNA that encodes for antibiotic resistance or herbicide resistance and cultivating the transformed tissue in the presence of the antibiotic or herbicide.

EXAMPLE 6

30 To make a transgenic marigold plant containing an isolated DNA that contains a DNA sequence from the beta-cyclase gene as shown in SEQ ID NO:1, marigold cotyledon tissue is transformed as in Example 4 or 5. The transformed tissue is used to make the transgenic plant. The DNA sequence produces RNA in the antisense orientation.

35

EXAMPLE 7

To make a transgenic marigold plant containing an isolated DNA that contains a DNA sequence from the beta-hydroxylase gene as shown in SEQ ID NO:3, marigold cotyledon
5 tissue is transformed as in Example 4 or 5. The transformed tissue is used to make the transgenic plant. The DNA sequence produces RNA in the antisense orientation.

EXAMPLE 8

10 To make a transgenic marigold plant containing an isolated DNA that contains a DNA sequence encoding the epsilon-cyclase gene as shown in SEQ ID NO:5, marigold cotyledon tissue is transformed as in Example 4 or 5. The transformed tissue is used to make the transgenic plant. The DNA sequence produces RNA in the antisense orientation.

15

EXAMPLE 9

To make a transgenic marigold plant containing an isolated DNA that contains a DNA sequence from the beta-cyclase gene as shown in SEQ ID NO:1, marigold cotyledon
20 tissue is transformed as in Example 4 or 5. The transformed tissue is used to make the transgenic plant. The DNA sequence produces RNA in the sense orientation which encodes beta-cyclase.

EXAMPLE 10

25

To make a transgenic marigold plant containing an isolated DNA that contains a DNA sequence from the beta-hydroxylase gene as shown in SEQ ID NO:3, marigold cotyledon tissue is transformed as in Example 4 or 5. The transformed tissue is used to make the transgenic plant. The DNA sequence produces RNA in the sense orientation which
30 encodes beta-hydroxylase.

EXAMPLE 11

To make a transgenic marigold plant containing an isolated DNA that contains a DNA
35 sequence encoding the epsilon-cyclase gene as shown in SEQ ID NO:5, marigold

cotyledon tissue is transformed as in Example 4 or 5. The transformed tissue is used to make the transgenic plant. The DNA sequence produces RNA in the sense orientation which encodes beta-cyclase.

5 EXAMPLE 12

To make a transgenic marigold plant containing an isolated DNA that contains a DNA sequence from the IPP isomerase gene as shown in SEQ ID NO:7, marigold cotyledon tissue is transformed as in Example 4 or 5. The transformed tissue is used to make the
10 transgenic plant. The DNA sequence produces RNA in the sense orientation which encodes IPP isomerase.

EXAMPLE 13

15 Transgenic marigold plants containing more than one isolated DNA containing a carotenoid biosynthesis synthesis gene in either the antisense or the sense orientation is made by cross-breeding the transgenic plants (made according to Examples 6 to 12) which contain isolated DNA containing the sequence from SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO 7, according to methods well known in the art such as those
20 provided in (Zhang et al, 1996; Zhong et al, 1996; Zhong et al, 1992). Transgenic plants that carry a low copy number of the isolated DNA used for cross-breeding.

Briefly, transgenic marigold plants that contain more than one isolated DNA are made by first making transgenic plants that contain either SEQ ID NO:1, SEQ ID NO:3, SEQ ID
25 NO:5 or SEQ ID NO:7 to make a first, a second, a third and a fourth transgenic plant. The first and second transgenic plants are cross-bred to create a bi-transgenic plant (contains SEQ ID NO:1 and SEQ ID NO:3) which can then cross-bred with the third transgenic plant to make a tri-transgenic plant which contains isolated DNAs containing SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5. The fourth transgenic plant can be crossed with the tri-
30 transgenic plant to produce the quadri-transgenic plant containing SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:7. In the above-described manner, transgenic plants containing any combination and any number of isolated DNAs can be constructed.

Transgenic plant lines containing more than one isolated DNA are cross-pollinated with
35 transgenic plant lines containing another isolated DNA. The resulting hybrid progeny are

cross-pollinated with transgenic plant lines containing other isolated DNAs. Each transgenic plant line produces specific carotenoid compounds depending on both what isolated DNAs are contained by the plant and whether the DNAs express RNA in the antisense orientation, the sense orientation or a combination thereof.

5

Alternatively, transgenic plants containing more than one type of isolated DNA can be made by multiple transformations. For example, cotyledon tissue from a transgenic plant containing one of the isolated DNAs can be transformed with another of the isolated DNAs to produce the bi-transgenic plant as shown in Examples 4 to 5.

10

Another alternative for making transgenic plants containing more than one type of isolated DNA is to either simultaneously transform the cotyledon tissue with multiple isolated DNAs containing the desired gene sequences or transform with one isolated DNA that contains each desired gene sequence. Transformation can be as shown as in Examples 4

15 to 5.

EXAMPLE 14

Transgenic marigold plants containing an isolated DNA which contains more than one
20 DNA sequence that produces antisense RNA to mRNA encoding at least two of beta-cyclase, beta-hydroxylase, or epsilon-cyclase are produced by a single transformation as shown in Example 4 or 5. The isolated DNA in this example contains DNA sequences from a combination of at least two DNA sequences selected from the group of DNA sequences which encodes beta-cyclase, beta-hydroxylase, or epsilon-cyclase wherein the
25 DNA sequences are in the antisense orientation.

EXAMPLE 15

Transgenic marigold plants containing an isolated DNA which contains more than one
30 DNA sequence that produces sense RNA encoding at least two of beta-cyclase, beta-hydroxylase, IPP isomerase or epsilon-cyclase are produced by a single transformation as shown in Example 4 or 5. The isolated DNA in this example contains DNA sequences from a combination of at least two DNA sequences selected from the group of DNA sequences which encodes beta-cyclase, beta-hydroxylase, IPP isomerase or epsilon-
35 cyclase.

EXAMPLE 16

Transgenic marigold plants containing an isolated DNA which contains at least one DNA sequence that produces sense RNA encoding at least one of beta-cyclase, beta-

5 hydroxylase, IPP isomerase, or epsilon-cyclase and at least one DNA sequence which produces antisense RNA to mRNA encoding at least one of beta-cyclase, beta-hydroxylase or epsilon-cyclase are produced by a single transformation as shown in Example 4 or 5. The isolated DNA in this example contains (1) a DNA sequence from at least one DNA sequence selected from the group of DNA sequences which encodes

10 beta-cyclase, beta-hydroxylase, or epsilon-cyclase, and (2) a DNA sequence in the antisense orientation from at least one DNA sequence not selected in (1).

While the present invention is described herein with reference to illustrated embodiments, it should be understood that the invention is not limited hereto. Those having ordinary skill

15 in the art and access to the teachings herein will recognise additional modifications and embodiments within the scope thereof.

REFERENCES

- Frey et al., (1995), Plant J. 8: 693-701.
- 20 James (1991), Antiviral Chem. Chemotherapy. 2: 191-214.
- Misawa et al., (1994). Plant J. 6: 481-489.
- Nellen et al., (1996). Mol. Biotechnol. 6: 7-15.
- Sambrook et al (1989). *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- 25 Senior (1998). Biotechnol. Genet. Rev. 15: 79-119.
- Yamaguchi-Shinozaki, et al., (1994). Plant Cell 6: 251-264.
- Zhang, et al., (1996). Theor. Appl. Genet., 92: 752-761.
- Zhong, et al., (1992). Planta 187: 483-489.
- Zhong, et al., (1996). Plant Physiology. 110: 1097-1107.

Applicant's or agent's file reference number	23166 PC 1	International application No.
--	------------	-------------------------------

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>15</u> , line <u>11</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
Name of depositary institution American Type Culture Collection	
Address of depositary institution (including postal code and country) 10801 University Blvd. Manassa, Va 20110-2209 USA	
Date of deposit 28 July 1999	Accession Number PTA-447
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

For receiving Office use only	
<input type="checkbox"/> This sheet was received with the international application	
Authorized officer	

For International Bureau use only	
<input type="checkbox"/> This sheet was received by the International Bureau on:	
Authorized officer	

INDICATIONS RELATING TO DEPOSITED MICROORGANISMS
(PCT Rule 12bis)

5 Additional sheet

In addition to the microorganism indicated on page 33 of the description, the following microorganisms have been deposited with

- 10 American Type Culture Collection, 10801 University Blvd.,
Manassas, VA 20110-2209, USA.

on the dates and under the accession numbers as stated below:

15

Accession number	Date of deposit	Description Page No.	Description Line No.
PTA-445	28 July 1999	15	19
20 PTA-446	28 July 1999	15	27
PTA-448	28 July 1999	15	35

- 25 For all of the above-identified deposited microorganisms, the following additional indications apply:

- As regards the respective Patent Offices of the respective designated states, the
- 30 applicants request that a sample of the deposited microorganisms stated above only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.

35

CLAIMS:

1. A transgenic plant material containing an isolated DNA encoding a marigold enzyme having catalytic activity of beta-cyclase.
- 5 2. The transgenic plant material according to claim 1 wherein the isolated DNA encodes a marigold enzyme having catalytic activity of an enzyme encoded by SEQ ID NO.:1.
3. The transgenic plant material according to claim 2 wherein the isolated DNA encodes
10 the enzyme from positions 304 to 1836 in SEQ ID. NO.:1.
4. The transgenic plant material according to claim 1 wherein the isolated DNA encodes a marigold enzyme having the amino acid sequence of SEQ ID NO.:2.
- 15 5. A transgenic plant material containing isolated DNA encoding a marigold enzyme having catalytic activity of beta-hydroxylase.
6. The transgenic plant material according to claim 5 wherein the isolated DNA encodes a marigold enzyme having catalytic activity of an enzyme encoded by SEQ ID NO.:3.
- 20 7. The transgenic plant material according to claim 6 wherein the isolated DNA encodes the enzyme from positions 51 to 923 in SEQ ID. NO.:3.
8. The transgenic plant material according to claim 5 wherein the isolated DNA encodes a
25 Marigold enzyme having the amino acid sequence of SEQ ID NO.:4.
9. A transgenic plant material comprising isolated DNA encoding a marigold enzyme having catalytic activity of epsilon-cyclase.
- 30 10. The transgenic plant material according to claim 9 wherein the isolated DNA encodes a marigold enzyme having catalytic activity of an enzyme encoded by SEQ ID NO.:5.
11. The transgenic plant material according to claim 10 wherein the isolated DNA encodes the enzyme from positions 141 to 1688 in SEQ ID. NO.:5.

12. The transgenic plant material according to claim 9 wherein the isolated DNA encodes a marigold enzyme having the amino acid sequence of SEQ ID NO.:5.
13. A transgenic plant material comprising one or more isolated DNAs encoding marigold
5 enzymes selected from the group consisting of beta-cyclase, beta-hydroxylase, epsilon-hydroxylase and epsilon-cyclase.
14. The transgenic plant material according to claim 13 wherein the beta-cyclase is encoded by SEQ ID NO.:1, the beta-hydroxylase is encoded by SEQ ID NO.:3 and the
10 epsilon-cyclase is encoded by SEQ ID NO.: 5.
15. The transgenic plant material according to claim 14 wherein the beta-cyclase is encoded by positions 304 to 1836 of SEQ ID NO.:1, the beta-hydroxylase is encoded by positions 51 to 923 of SEQ ID NO.:3 and the epsilon-cyclase is encoded by positions 141
15 to 1688 of SEQ ID NO.: 5.
16. The transgenic plant material according to claim 15 wherein the beta-hydroxylase has the amino acid sequence of SEQ ID NO.:2, the beta-cyclase has the amino acid sequence of SEQ ID NO.:4 and the epsilon-cyclase has the amino acid sequence of SEQ
20 ID NO.: 6.
17. An isolated DNA comprising a DNA sequence encoding marigold beta-cyclase.
18. The isolated DNA according to claim 17 having the DNA sequence of SEQ ID NO.:1.
25
19. The isolated DNA according to claim 18 wherein the DNA sequence encodes beta-cyclase having the amino acid sequence of SEQ ID NO.:2.
20. An isolated DNA comprising a DNA sequence encoding marigold beta-hydroxylase.
30
21. The isolated DNA according to claim 20 having the DNA sequence of SEQ ID NO.:3.
22. The isolated DNA according to claim 21 wherein the DNA sequence encodes beta-hydroxylase having the amino acid sequence of SEQ ID NO.:4.
35

23. An isolated DNA comprising a DNA sequence encoding marigold epsilon-cyclase.

24. The isolated DNA according to claim 23 having the DNA sequence of SEQ ID NO.:5.

5 25. The isolated DNA according to claim 24 wherein the DNA sequence encodes epsilon-cyclase having the amino acid sequence of SEQ ID NO.:6.

26. A transgenic plant material containing at least one isolated DNA which produces an RNA that is antisense to a marigold enzyme selected from the group consisting of beta-cyclase, beta-hydroxylase, epsilon-hydroxylase and epsilon-cyclase.
10

27. The transgenic plant material according to claim 26 wherein the isolated DNA is selected from the group consisting of beta-cyclase encoded by SEQ ID NO.:1, beta-hydroxylase encoded by SEQ ID NO.:3 and epsilon-cyclase encoded by SEQ ID NO.:5.
15

28. The transgenic plant material according to claim 27 wherein the isolated DNA is selected from the group consisting of beta-cyclase encoded by positions 304 to 1836 of SEQ ID NO.:1, beta-hydroxylase encoded by positions 51 to 923 of SEQ ID NO.:3 and epsilon-cyclase encoded by positions 141 to 1688 of SEQ ID NO.:5.
20

29. A transgenic plant material containing at least one isolated marigold DNA sequence selected from the group consisting of a DNA sequence encoding an enzyme having catalytic activity of beta-hydroxylase, a DNA sequence encoding an enzyme having catalytic activity of beta-cyclase, a DNA sequence encoding an enzyme having catalytic activity of epsilon-cyclase, a DNA sequence encoding an enzyme having catalytic activity of epsilon-hydroxylase and a DNA sequence encoding an enzyme having catalytic activity of IPP isomerase wherein a first end of the DNA sequence is operably linked to a RNA promoter and a second end of the DNA sequence is operably linked to a regulatory sequence containing a polyadenylation signal, such that upon transformation, the plant
25
30 material produces the enzyme encoded by the isolated DNA sequence.

30. A transgenic plant material containing at least one isolated marigold DNA sequence selected from the group consisting of a DNA sequence encoding an enzyme having catalytic activity of beta-hydroxylase, a DNA sequence encoding an enzyme having catalytic activity of beta-cyclase, a DNA sequence encoding an enzyme having catalytic
35

activity of epsilon-cyclase, and a DNA sequence encoding an enzyme having catalytic activity of epsilon-hydroxylase wherein a first end of the DNA sequence is operably linked to a RNA promoter and a second end of the DNA sequence is operably linked to a regulatory sequence containing a polyadenylation signal, such that upon transformation,
5 the plant material produces an RNA antisense to a mRNA produced by the plant.

31. A transgenic plant material containing at least two marigold DNA sequences selected from the group consisting of a DNA sequence encoding an enzyme having catalytic activity of beta-hydroxylase, a DNA sequence encoding an enzyme having catalytic
10 activity of beta-cyclase, a DNA sequence encoding an enzyme having catalytic activity of epsilon-cyclase, a DNA sequence encoding an enzyme having catalytic activity of epsilon-hydroxylase and a DNA sequence encoding an enzyme having catalytic activity of IPP isomerase wherein a first end of the DNA sequence is operably linked to a RNA promoter, and wherein at least one DNA sequence produces an RNA in an orientation antisense to
15 a mRNA and remaining DNA sequence produces an RNA in a sense orientation such that upon transformation, the plant material produces an RNA molecule from the first recombinant DNA construct antisense to the mRNA produced by the plant and produces the enzyme encoded by the second recombinant DNA construct.

20 32. The transgenic plant according to any one of claims 29, 30 or 31, wherein the DNA sequence encoding the beta-cyclase is encoded by positions 304 to 1836 of SEQ ID NO.1, the DNA sequence encoding beta-hydroxylase is encoded by positions 51 to 923 of SEQ ID NO.:3, and the DNA sequence encoding the epsilon-cyclase is encoded by positions 141 to 1688 of SEQ ID NO.:5.

25

33. The transgenic plant according to any one of claims 29, 30 or 31 wherein the RNA promoter is a petal specific promoter.

34. The transgenic plant according to claim 33 wherein the RNA promoter is a promoter
30 for a ketolase gene from *Arabidopsis thaliana*.

35. The transgenic plant according to claim 34 wherein the RNA promoter is a promoter for a ketolase gene from *Adonis vernalis*.

36. A method for manipulating carotenoid synthesis in a plant material, the steps comprising:

5 (a) providing at least one isolated marigold DNA sequence selected from the group consisting of a DNA sequence encoding an enzyme having catalytic activity of beta-hydroxylase, a DNA sequence encoding an enzyme having catalytic activity of beta-cyclase, a DNA sequence encoding an enzyme having catalytic activity of epsilon-cyclase, a DNA sequence encoding an enzyme having catalytic activity of epsilon-hydroxylase, a DNA sequence encoding an enzyme having
10 catalytic activity of IPP isomerase wherein a first end of the DNA sequence is operably linked to a RNA promoter and a second end of the DNA sequence is operably linked to a regulatory sequence containing a polyadenylation signal;

15 (b) transforming plant material with the isolated DNA; and

(c) isolating the plant.

37. The method according to claim 36 wherein at least one isolated DNA is operably linked to the RNA promoter to produce an RNA that is antisense to an mRNA.

20

38. The method according to claim 36 wherein at least one isolated DNA is operably linked to the RNA promoter to produce the enzyme encoded by the isolated DNA sequence.

25 39. The transgenic plant of any one according to claims 36, 37 or 38 wherein the RNA promoter is a petal specific promoter.

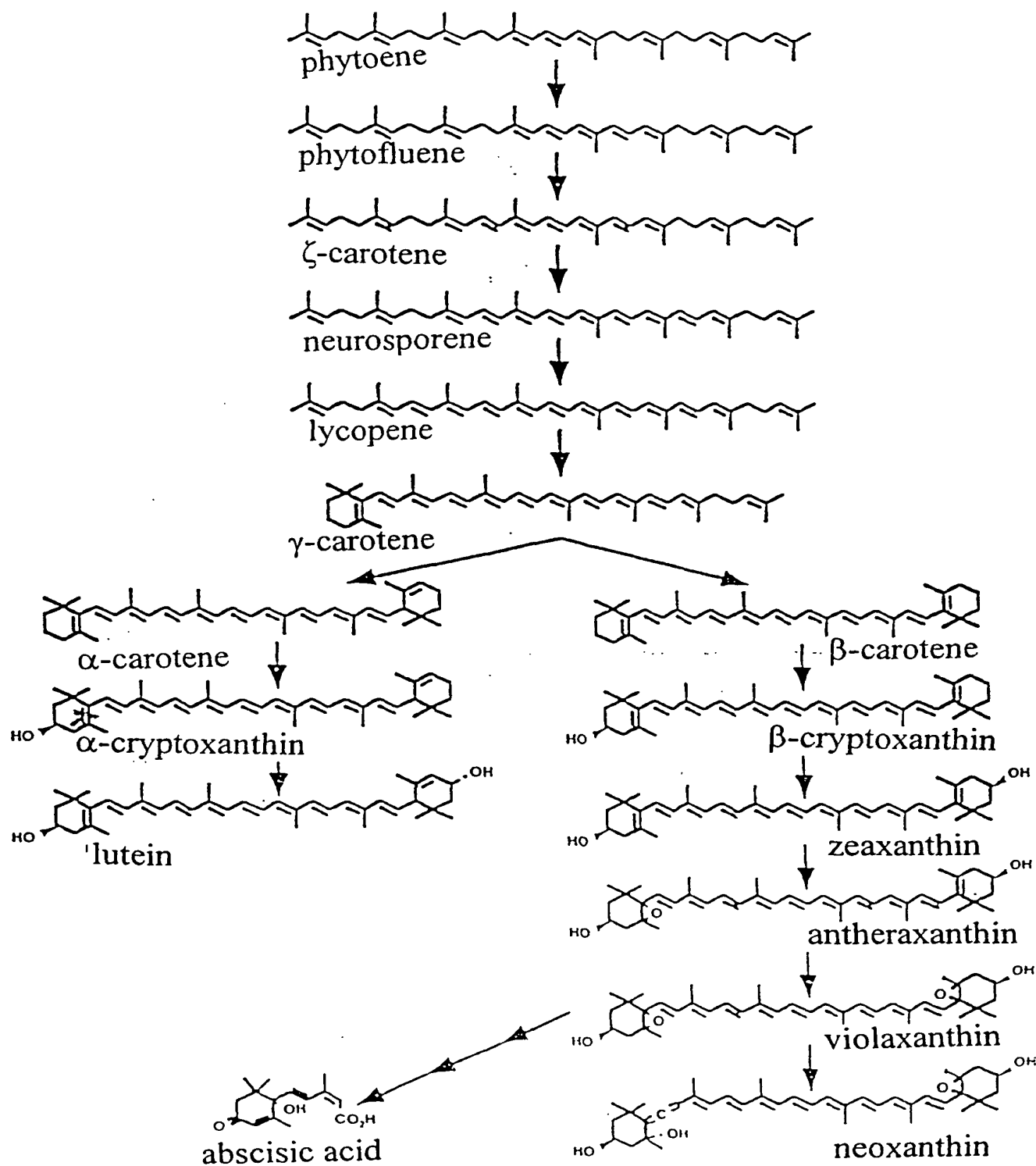
40. The transgenic plant according to claim 39 wherein the RNA promoter is a promoter for a ketolase gene from *Adonis vernalis*.

30

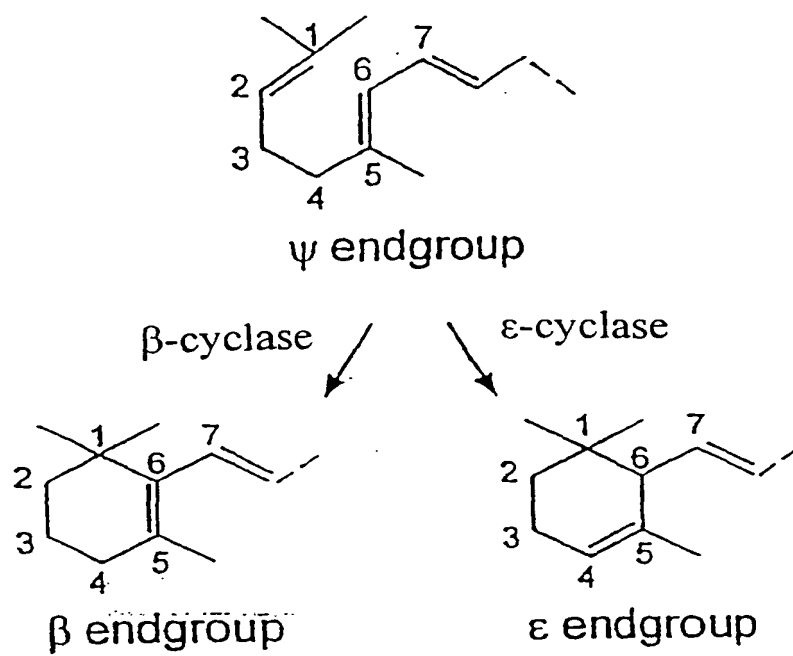
41. A transgenic plant material containing an isolated DNA encoding a marigold enzyme having catalytic activity of IPP isomerase.

42. The transgenic plant material according to claim 41 wherein the isolated DNA encodes a marigold enzyme having catalytic activity of an enzyme encoded by SEQ ID NO.:7.
- 5 43. The transgenic plant material according to claim 42 wherein the isolated DNA encodes the enzyme from positions 101 to 796 in SEQ ID. NO.:7.
44. The transgenic plant material according to claim 43 wherein the isolated DNA encodes a marigold enzyme having the amino acid sequence of SEQ ID NO.:8.
- 10 45. An isolated DNA comprising a DNA sequence encoding marigold IPP isomerase.
46. The isolated DNA according to claim 45 having the DNA sequence of SEQ ID NO.:7.
47. The isolated DNA according to claim 46 wherein the DNA sequence encodes beta-
- 15 cyclase having the amino acid sequence of SEQ ID NO.:8.

1/10



2/10



3/10

TCTAGAACTAGTGGATCCCCGGGCTGCAGGAATTCCGGCACGAGACTTCCCATTATCC
AATCTCTCAAAACCATCAACAAATTCACCACATCATTTACCGTAAGTCITCATATCTTT
CAATCTTTCACAAACCCACTTCAATCTCATCATTAATCTCATAAAGTTTCATACCTTTGTT
GTCAATTTTGGTGTCTTGGTTCATGATTCATAAAGTTCATAACITTTGTTGCTGTTTTT
GTGTTCTTGATTCAAAAGTTCAAAACITTTGTTGTTTTTTTGTTAAATTACATCTGGG
TTTTCATGGATACCTTCTTAAGAACATACAAATTCGTTTGAATTTGTGCACCCCAAGTAACAA
ATTGACAGGAAATTTGAACAAATTTGAATCAATGAATCAATCAAAAGTCTCAATTTCAAG
ACTTAGATTTGGCCCAAAATAATCCCAATTCAAAATTAGGGCAAAATATTGTTAAAG
GCTAGTAGTAGTGTCTTTAGAACTTGTCTGAAATCAAAAGAAATCTTGATTT
TGATCTTCTATGTATGATCCATCAAGAAATGTTGTGGTGATCTTGTGGTTGGTG
GTGTCCTTCAGGGTTAGCAGTGGCTCAACAAGTGTCTGAGGCTGGTCTCACAGTGTG
CTCAATTGACCCATCACCTAAACTCATTTGGCCCAATAATTATGGTGTGGTGTGATG
AGTTTGAAGCTATGGATTTGTTGGATTGTTGGATACAACTTGGTCAAGTGTGTTGTT
TACATTTGATGAAAGTCAACCAAGAGTCTTAATAGACCATATGCAAGAGTCAATAGAA
AACAACTTAAACAAAGATGTTACAAAGTATAGCAATGGGTTAAGTTTCATCAA
GCAAAAGTCAATCAAGTATTCATGAAGAGTTAAATCTTTGTTGATTTGTAATGATGG
TGTCACAAATTCAGCCACTTGTGTTCTTGATGCACTGGTTTTCAAGATCTTTAGTTCA
ATATGATAAGCCTTATAACCTGGGTACCAAGTGGCTTATGGATTTAGCCGAAGTT
GAAGAACACCCCTTTTGACGTTGATAAATGTTGTTTATGGATTGGAGAGATTCACACCT
TGATCAAAATCTTGAAATTAAGCTAGAAATTCAGAAATCCCAACTTTTATACGGGAT
GCCATTTTCGTCTACAAGAACTCTTCTTGAAGAAACATCACTGTTGCTCGTCCGGGGT
TGAAGATGGAAGATATTCAAGAAAGATGGCTTACAGGCTAAGCATTTGGGGATAAA
AGTAAAGCATTTGAAGAACGACGAGTTGTGTTATCCCGATGGCGGGCCCCCTACC
AGTGCTCCCTCAACGGTTCTTGGATAGGTGGTACAGCAGGAATGGTGCATCCGTCA
ACCGGATACATGGTGGCAAGACGCTAGCAGCCGCCCGATTGTTGCAAAAGTCAATA
ATCCGGTATCTTAATAACGAAAAAGTATGGTGGCCGACGTCACCGGAGATGATTTAG
CAGCCGGAATATGGAGAGAAATTTGGCCCTATTGAAGAGAGGAGACAAGGGAGTTTT
TTTGTTTGGGATGGATATATTGTTGAAGCTTGATTGGAAAGGTACTAGAGGTTCTTT
GATCGGTTTTTCGACTTGGAACTCGTTATTGGCATGGGTTTTTGTCTCGAGGTTGTT
TCTACCGGAGTTAGTGACGTTTGGGCTATCGCTTTTCGGTCAATGCTTCGAATACITGTA
GAGTTGAAATTAATGGCAAAAGGGAATCTTCCATTGGCAACTATGATGGTAAATTTGGTT
AGAGATGGAGAAATGAATTAATGAATATCAAGATTAAATATAGTTATTTATATATCTTG
TATGCTTTCAGTTTTTGTAAATTGGATGTTATGGTAAATTTGATGTTTTAAGTTGATTAA
AAAAAAA

Fig. 3

4/10

MDTFLRTYNSFEFVHPSNKFAGNLLNQLNQSKSQFDFFRFGPKKSQFKLGQKYCVK
ASSALLELVPEIKKENLDFDLPMYDPSRNVVVDLVVVGPGPSGLAVAQQVSEAGLTVCSI
DSPKLIWPNNGVWVDEFEAMDLLDCLDTTWSSAVVYDEKSTKSLNRPYARVNRKQL
KTKMLQKCLANGVKFHQAKVIKVIHEELKSLLICNDGVTIQA TLVLDA TGFSRSLVQYDKP
YNPGYQVAYGILAEVEEHPFDVDKMLFMDWRDSDLQNLKARNRPTFLYAMPFSST
RIFLEETSLVARPGLKMEIQERMA YRLKHLGIKVKSEEDERCVIPMGGPLPVLPRVLGI
GGTAGMVHPSTGYMVARTLAAPIVAKSIIRYLNNEKSMVADVTGDDLAAGIWRELWPPIE
RRRQREFFCFGMDILLKLDLEGTRRFFDAFFDLEPRYWHGFLSSRLFLPELVTFGLSLFGH
ASNTCRVEIMAKGTPLATMIGNLVDRRE

Fig. 4

5/10

GGCACGAGGCAAGCAAAGGTTGTTGTTGTTGAGAGACACTCCAATCCAAA
CAGATACAAGGCGTACTGGATATTTCTCTCTCGTTCCTAACACAGCAACGAAGAAG
AAAAGAATCATTAATAACAATCAATGAGTATGAGAGCTGGACACATGACGGCAACAA
TGGCGGCTTTACATGCCCTAGGTTTATGACTAGCATCAGATACACGAAGCAAATTAA
GTGCAACGCTGCTAAAGCCAGCTAGTCGTTAAACAAGAGATTGAGGAGGAAGAAGA
TTATGTGAAGCCGGTGGATCGGAGCTGCTTTTGTTCAAATGCAACAGAAATAGTCC
ATGATGCACAGTCTAGCCTATCCCAAAAGCTCCCAAGGTACCAATAGGAGGAGGA
GGAGACAGTAAGTCTGATGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTG
CTCTTGTGGAGAAATCAGCCAAAGCTAGGCTTGAATGTCGCACTTATCGGCCCTGATCT
TCCCTTTACAAATACTATGGTGTGGGAGGATGAATTTATAGGTCTTGGACTTGAGG
GCTGTATTGAACAATGTTGGCGAGATACTGTAGTATATCTTGATGACAAACGATCCCAAT
CTCATAGGTCGTGCTATGGACGAGTTAGTCGTGATTTACTTCACGAGGAGTTGTGA
CTAGGTGCATGGAGTCAGGCGTTTCATATCTGAGCTCCAAAGTGAACGGATTACTGA
AGTCCCAAATGGCCTAAGTCTCATAGAGTGAAGGCAATATCACAATTCATGCAGG
CTTGCTACTGTGCTTCTGGAGCAGCTTCTGGAAAACTTTTGCAATATGAACTTGGCG
GTCCCCGTGTTGCGTTCAAAACAGCTTATGGTATAGAGTTGAGGTTGAAGCATACC
CTATGATCCAAGCCTAAATGGTTTTCATGGATTATAGAGACTACACCAACATAAATCTC
AATCACTAGAAGCACAAATATCCAACATTTTGTATGTATGTCATGCCAATGTCTCCAACATAA
GTATTTCTTGAGGAACTGTTGGCTTCAAAAGAGGCCATGCCCTTTTGAGTTATTGAA
GACAAACTCATGTCAAGATTAAAGACTATGGGATCCGAATACCAAACTTATGAA
GAGGAATGGTCATATATCCAGTAGGTGGATCCTTACCAATACCGAGCAAAAGAACCC
TTGCAATTTGGTGTCTGCTAGCATGGTGCTATCCAGCCACAGGATATTCCGTTGTAA
ATCACTGTCAGAAAGCTCCTAATTATGCAGCAGTAATTGCAAGATTTTAGGGAAGGA
AATTCAAACACAGATGCTTGATCATGGAAGATACACAACCAACATCTCAAAGCAAGCTT
GGGAACACTTTGGCCCCCTTGAAGGAAAGACAGAGAGCATTTCTTCTCTTTGGATT
AGCACTGATTGTCCAGATGGATATTGAGGGGACCCGCACATTTCTCCGGACTTTCTTC
CGCTTGCCCATGGATGTGGTGGGGTTCTTGGATCTTCGTTATCATCAACTGACTT
GATAATATTGGGTTTACATGTTTATCATAGCACCGCATAGCCTGAGATGGGTCTGG
TTAGACATTTGCTTCTGACCCGACAGGAGGAACAATGTTAAAGCGTATCTCACGATA
TAAATAACTCTAGTCGGATCAGTTTAGATTATAGGCACATCTTGCCATATATATGTAT
AAACCTTATGTGTGTATCCTTACATCAACACAGTCAATTAATTGTATTTCTTGGGGTA
ATGCTGATGAAGTATTTTCTGGAAAAAATAAAAAAATACTCGAGACTAGTTCACCTCT
CTCTCTCTCTGCGCGATTCT

Fig. 5

6/10

MSMRAGHMTATMAAFTCPRFMTSIRYTKQIKCNAAKSQLVVKQIEIEEDYVKAGGSELL
 FVQMQQNKSMDAQSSLSQKLPRVPIGGGDSNCILDLVIGCGPAGLALAGESAKLGLNV
 ALIGPDLPTNNYGVWEDEFGLGLEGCIEHVWRD TVVYLDNDPILIGRAYGRVSRDLLH
 EELLTRCMESGVSYLSSKVERITEAPNGLSIECEGNITPCRLATVASGAASGKLLQYELG
 GPRVCVQTAYGIEVEVESIPYDPSLMVFMDYRDYTKHKSQSLEAQYPTFLYVMPMSPTKV
 FFEETCLASKEAMPFELLKTKLMSRLKTMGIRITKTYEEWSYIPVGGSLPNTQKNLAFG
 AAASMVHPATGYSVVRSLSEAPNYAAVIAKILGKNSKQMLDHGRYTTNISKQAWETLW
 PLERKRQRAFFLFGLALIVQMDIEGTRTFFRTFTWMWWGFLGSSLSSTDLIIFAFYMF
 IAPHSLRMGLVRHLLSDPTGGTMLKAYLTI

Fig. 6

7/10

GGCACGAGATTGCTGTCCCTTGTAGCTCAAGACCAATTTGGCTTAGGTCGAATGCGGTT
ACTTGGTCATAAACCCACAACCATAACTTGTCACCTCCCTTTTCCTTTCTATCAAAATC
ATTTACCCCAATTGTTAGGGCAGAAGATGTACTGTTGTTGTTGTTGCCGTTGCCGAC
AGTAATAGTAACAGTAATAATAAGTGACAGTAATAGTAATAATCCGGGTTCTGGATTT
AAACCCGGCGTTATGAACCGTAACCGTTTGGTTGAAGAAATAATGGAGAGGAAATAA
ATCGGAACGATTTACTTATCTTGTGTCAGCTATTATGTCTACTTTTGGAAATTACTTCAAT
GGCGGTTATGGCGGTTTATTACCGGTTTTCATGGCAAATGGAGGGTGAGAAATTCCT
TATGTGGAGATGTTTGGTACATTTGCTCTCTCCGTTGGTGGTGGAGGAGGAGTGGAGT
ATTGGGCAAGATGGGCTCATGAGGCACTATGGCATGCTTCTTTGTGGCACATGCATGA
GTCACACCATAAAGCCACGAGAAAGGTCCGTTTGAGCTTAATGATGTGTTGCTATAACA
AATGCGGTCCCGGCCATTGCGTTGCTTAGTTATGGGTTTTCACAAAGGCATAATTCC
GGGTCCTTTGTTTGGGCGGACTGGGAATTACGGTGTGTTGGAAATGGCGTATATGTTT
GTCCACGACGGGCTAGTTACAGAAAGATTCCAAAGTGGTCCGATTGCCGAATGTTCCCT
ATCTTCGAAGGGTTGCAGCGGCTCATCAGTGCATCACACGGAAATAATTAATGGTGT
TCCCTTATGGCTTGTCTTGGACCTAAGGAGCTAGAAGAAGTGGGTGTCGGGAAGA
ATTGGACAAGGAGATTCAAAGAAGAAATTAATGATAATAATACTAAATAATAATT
TTGTATAAAATTAAATAATTAATGATATCTTTTGTAAAAAATAAAAAA

Fig. 7

8/10

MRLLGHKPTTITCHEPFSFSIKSFTPIVRGRRCTVCFVAGGDSNSNSNNSDSNSNPNGL
DLNPAVMNRNRLVEEKMERKKSERFTYLVAAMSTFGITSMAYMAVYYRFSWQMEGGEI
PYVEMFGTTFALSVGAAVGMEYWARWAHEALWHASLWHMHESHKPREGPFELNDVFA
ITNAVPAIALLSYGFFHKGIIPGLCFGAGLGITVFGMAYMFVHDGLVHRRFQVGPIANVPY
LRRVAAAHQLHTEKFNGVPYGLFLGPKELEEVGTEELDKEIQRRIKLYNNTK

Fig. 8

9/10

CAGGAATTGGGACGAGCTCAATCTCAATCAACCCCTCTTCTCTCTCCCAAGTATCTATA
CCAAACAACCTCAATCTCTCCGTCGCTCTTACTCCGCCATGGGTGACGACTCCGG
CATGGATGCTGTTACGGACGTCATGTTAACGATGAATGCATTTTGGTGGATGAG
TGTGACAAATGTGGTGGACATGATACCAATACAATTGTCACTTGATGGAGAGATTG
AAACAGGTAAATGCTGCACAGAGCATTCAGCGTTTTTCTATTCAATTCAAAATACGAG
TTACTTCTTCAGCAACGGTCTGCAACCAAGGTGACATTTCCCTTAGTATGGACCAACAC
CTGTTGCAGCCATCCACTCTACAGAGAACTCCGAGCTTGTCCCGAAACGCCCTTGG
GTAAGAAATGCTGCACAGAGGAAGCTGTTGGATGAACCTGGTATCCCTGCTGAAGAT
GTTCCCGTTGATCAGTTTACTCCTTAGGTGCGATGCTCTACAAGGCTCCAATCTGATGG
AAAGTGGGGAGAACATGAACCTTGACTACCTACCTTTTCAATAGTGAGAGACGTTGCTGTA
AACCCGAACCCAGATGAAGTGGCGGATATCAATAATGTGAACCAAGAGATTAAAG
GAGCTGCTAAGGAAGCAGATGCGGGGAGGAGGTTTGAAGCTGTCTCCATGGTTC
AGGTTAGTGGTTGATAACTTCTTGTTCAGTGGTGATCATGTGCAAAAGGTTACAC
TCACTGAAGCAATTGATATGAACAACCATACACAAGCTGATATAGAAACACACCCCTCAAC
CGAAAGTTCAAGCCTAATAATTGGGTTGGTGGGCTCTACCATCAATTGTTTTTC
TTTTAAGAAAGTTTTAACTCTAATTTGAGCATGTTGATTTCTGTTTGTGTAAGATT
TGGGTTTCGTTTCAGTTGTAAATGAACCATTGATGGTTTGCAATTTCAGTTCCTATC
GACAAAAAATAAAAAAACTC

Fig. 9

10/10

MGDDSGMDA VQRRLMFNDECILVDECDNVVGHDTKYNCIHLMEKIETGKMLHRAFSVFL
FNSKYELLQQRSATKVTFFPLVWNTTCCSHPLYRESELVPENALGVRNAAQRKLLDELGIP
ALEDVPVDQFTPLGRMLYKAPSDGKWGEHELDYLLFIVRDVA VNPDPDEVADIKYVYNQEEL
KELLRKADAGEEGLKLSPWFRLLVVDNFLFKWWDHVQKVTLTEAIDMKTIHKL

Fig. 10

SEQUENCE LISTING

<110> Cunningham Jr, Francis X
DellaPenna, Dean

5 <120> Method for Regulating Carotenoid Biosynthesis in
Marigolds

<130> Chr. Hansen A/S

10 <140> 09/201,641
<141> 1998-11-30

<160> 8

15 <170> PatentIn Ver. 2.0

<210> 1
<211> 1959
<212> DNA

20 <213> Tagetes erecta

<220>
<221> CDS
<222> (304)..(1836)

25 <223> beta-cyclase

<400> 1
tctagaacta gtggatcccc cgggctgcag gaattcggca cgagacttcc cattatccaa 60

30 tctctcaaaa ccatcaacaa tttcaccaca tcatttaccg gtaagtcttc atatctttca 120
attcttcaca aaccacttcc aattctcatc attaatctca taaagttcat acctttgttg 180
tcaatttttg tgtttcttgg gttcttgatt cataaagttc ataactttgt tgctgttttt 240

35 gtgtttcttg attcataaag ttcaaaattt gttggttttt ttgttaaatt acatctgggt 300

40 ttc atg gat acc ttc tta aga aca tac aat tgc ttt gaa ttt gtg cac 348
Met Asp Thr Phe Leu Arg Thr Tyr Asn Ser Phe Glu Phe Val His
1 5 10 15

45 cca agt aac aaa ttt gca gga aat ttg aac áat ttg aat caa ttg aat 396
Pro Ser Asn Lys Phe Ala Gly Asn Leu Asn Asn Leu Asn Gln Leu Asn
20 25 30

50 caa tca aag tct caa ttt caa gac ttt aga ttt ggc cca aaa aaa tcc 444
Gln Ser Lys Ser Gln Phe Gln Asp Phe Arg Phe Gly Pro Lys Lys Ser
35 40 45

55 caa ttc aaa tta ggg caa aaa tat tgt gtt aaa gct agt agt agt gct 492
Gln Phe Lys Leu Gly Gln Lys Tyr Cys Val Lys Ala Ser Ser Ser Ala
50 55 60

60 ttg tta gaa ctt gtt cct gaa atc aag aaa gaa aat ctt gat ttt gat 540
Leu Leu Glu Leu Val Pro Glu Ile Lys Lys Glu Asn Leu Asp Phe Asp
65 70 75

65 ctt cct atg tat gat cca tca aga aat gtt gtg gtg gat ctt gtg gtg 588
Leu Pro Met Tyr Asp Pro Ser Arg Asn Val Val Val Asp Leu Val Val
80 85 90 95

70 gtt ggt ggt ggt cct tca ggg tta gca gtg gct caa caa gtg tct gag 636
Val Gly Gly Gly Pro Ser Gly Leu Ala Val Ala Gln Gln Val Ser Glu
100 105 110

	gct ggt ctc aca gtg tgc tca att gac cca tca cct aaa ctc att tgg	684
	Ala Gly Leu Thr Val Cys Ser Ile Asp Pro Ser Pro Lys Leu Ile Trp	
	115 120 125	
5	ccc aat aat tat ggt gtt tgg gtt gat gag ttt gaa gct atg gat ttg	732
	Pro Asn Asn Tyr Gly Val Trp Val Asp Glu Phe Glu Ala Met Asp Leu	
	130 135 140	
10	ttg gat tgt ttg gat aca act tgg tca agt gct gtt gtt tac att gat	780
	Leu Asp Cys Leu Asp Thr Thr Trp Ser Ser Ala Val Val Tyr Ile Asp	
	145 150 155	
15	gaa aag tca acc aag agt ctt aat aga cca tat gca aga gtc aat aga	828
	Glu Lys Ser Thr Lys Ser Leu Asn Arg Pro Tyr Ala Arg Val Asn Arg	
	160 165 170 175	
	aaa caa ctt aaa aca aag atg tta caa aag tgt ata gca aat ggg gtt	876
	Lys Gln Leu Lys Thr Lys Met Leu Gln Lys Cys Ile Ala Asn Gly Val	
	180 185 190	
20	aag ttt cat caa gca aaa gtc atc aaa gtg att cat gaa gag tta aaa	924
	Lys Phe His Gln Ala Lys Val Ile Lys Val Ile His Glu Glu Leu Lys	
	195 200 205	
25	tct ttg ttg att tgt aat gat ggt gtc aca att caa gcc act ttg gtt	972
	Ser Leu Leu Ile Cys Asn Asp Gly Val Thr Ile Gln Ala Thr Leu Val	
	210 215 220	
30	ctt gat gca act ggt ttt tca aga tct tta gtt caa tat gat aag cct	1020
	Leu Asp Ala Thr Gly Phe Ser Arg Ser Leu Val Gln Tyr Asp Lys Pro	
	225 230 235	
35	tat aac cct ggg tac caa gtg gct tat ggg att tta gcc gaa gtt gaa	1068
	Tyr Asn Pro Gly Tyr Gln Val Ala Tyr Gly Ile Leu Ala Glu Val Glu	
	240 245 250 255	
	gaa cac cct ttt gac gtt gat aaa atg ttg ttt atg gat tgg aga gat	1116
	Glu His Pro Phe Asp Val Asp Lys Met Leu Phe Met Asp Trp Arg Asp	
	260 265 270	
40	tca cac ctt gat caa aat ctt gaa att aaa gct aga aat tca aga atc	1164
	Ser His Leu Asp Gln Asn Leu Glu Ile Lys Ala Arg Asn Ser Arg Ile	
	275 280 285	
45	cca act ttt tta tac gcg atg cca ttt tcg tct aca aga atc ttt ctt	1212
	Pro Thr Phe Leu Tyr Ala Met Pro Phe Ser Ser Thr Arg Ile Phe Leu	
	290 295 300	
50	gaa gaa aca tca ctc gtt gct cgt ccg ggg ttg aag atg gaa gat att	1260
	Glu Glu Thr Ser Leu Val Ala Arg Pro Gly Leu Lys Met Glu Asp Ile	
	305 310 315	
55	caa gaa aga atg gct tac agg cta aag cat ttg ggg ata aaa gta aaa	1308
	Gln Glu Arg Met Ala Tyr Arg Leu Lys His Leu Gly Ile Lys Val Lys	
	320 325 330 335	
	agc att gaa gaa gac gaa cgt tgt gtt atc ccg atg ggc ggg ccc cta	1356
	Ser Ile Glu Glu Asp Glu Arg Cys Val Ile Pro Met Gly Gly Pro Leu	
	340 345 350	
60	cca gtg ctc cct caa cgg gtt ctt gga ata ggt ggt aca gca gga atg	1404
	Pro Val Leu Pro Gln Arg Val Leu Gly Ile Gly Gly Thr Ala Gly Met	
	355 360 365	
65	gtg cat ccg tca acc gga tac atg gtg gca aga acg cta gca gcc gcc	1452

Val His Pro Ser Thr Gly Tyr Met Val Ala Arg Thr Leu Ala Ala Ala
 370 375 380
 ccg att gtt gca aag tca ata atc cgg tat ctt aat aac gaa aaa agt 1500
 Pro Ile Val Ala Lys Ser Ile Ile Arg Tyr Leu Asn Asn Glu Lys Ser
 385 390 395
 5
 atg gtg gcc gac gtc acc gga gat gat tta gca gcc gga ata tgg aga 1548
 Met Val Ala Asp Val Thr Gly Asp Asp Leu Ala Ala Gly Ile Trp Arg
 400 405 410 415
 10
 gaa ttg tgg cct att gaa aga agg aga caa agg gag ttt ttt tgt ttt 1596
 Glu Leu Trp Pro Ile Glu Arg Arg Arg Gln Arg Glu Phe Phe Cys Phe
 420 425 430
 15
 ggg atg gat ata ttg ttg aag ctt gat ttg gaa ggt act aga agg ttc 1644
 Gly Met Asp Ile Leu Leu Lys Leu Asp Leu Glu Gly Thr Arg Arg Phe
 435 440 445
 20
 ttt gat gcg ttt ttc gac ttg gaa cct cgt tat tgg cat ggg ttt ttg 1692
 Phe Asp Ala Phe Phe Asp Leu Glu Pro Arg Tyr Trp His Gly Phe Leu
 450 455 460
 25
 tcg tcg agg ttg ttt cta ccg gag tta gtg acg ttt ggg cta tcg ctt 1740
 Ser Ser Arg Leu Phe Leu Pro Glu Leu Val Thr Phe Gly Leu Ser Leu
 465 470 475
 30
 ttc ggt cat cgt tcg aat act tgt aga gtt gaa att atg gca aaa ggg 1788
 Phe Gly His Arg Ser Asn Thr Cys Arg Val Glu Ile Met Ala Lys Gly
 480 485 490 495
 35
 act ctt cca ttg gca act atg att ggt aat ttg gtt aga gat cga gaa 1836
 Thr Leu Pro Leu Ala Thr Met Ile Gly Asn Leu Val Arg Asp Arg Glu
 500 505 510
 40
 tgaataattg aatatcaaga ttaatttata gttatttata tatacttgta tgctttcagt 1896
 ttttgtaaat tggatgttat ggtaattgta tgttttaagt tgattaaaaa aaaaaaaaaa 1956
 aaa 1959
 45
 <210> 2
 <211> 511
 <212> PRT
 <213> Tagetes erecta
 50
 <400> 2
 Met Asp Thr Phe Leu Arg Thr Tyr Asn Ser Phe Glu Phe Val His Pro
 1 5 10 15
 55
 Ser Asn Lys Phe Ala Gly Asn Leu Asn Asn Leu Asn Gln Leu Asn Gln
 20 25 30
 Ser Lys Ser Gln Phe Gln Asp Phe Arg Phe Gly Pro Lys Lys Ser Gln
 35 40 45
 60
 Phe Lys Leu Gly Gln Lys Tyr Cys Val Lys Ala Ser Ser Ser Ala Leu
 50 55 60
 65
 Leu Glu Leu Val Pro Glu Ile Lys Lys Glu Asn Leu Asp Phe Asp Leu
 65 70 75 80
 Pro Met Tyr Asp Pro Ser Arg Asn Val Val Val Asp Leu Val Val Val
 85 90 95
 65

Gly Gly Gly Pro Ser Gly Leu Ala Val Ala Gln Gln Val Ser Glu Ala
 100 105 110
 Gly Leu Thr Val Cys Ser Ile Asp Pro Ser Pro Lys Leu Ile Trp Pro
 115 120 125
 5 Asn Asn Tyr Gly Val Trp Val Asp Glu Phe Glu Ala Met Asp Leu Leu
 130 135 140
 Asp Cys Leu Asp Thr Thr Trp Ser Ser Ala Val Val Tyr Ile Asp Glu
 145 150 155 160
 10 Lys Ser Thr Lys Ser Leu Asn Arg Pro Tyr Ala Arg Val Asn Arg Lys
 165 170 175
 15 Gln Leu Lys Thr Lys Met Leu Gln Lys Cys Ile Ala Asn Gly Val Lys
 180 185 190
 Phe His Gln Ala Lys Val Ile Lys Val Ile His Glu Glu Leu Lys Ser
 195 200 205
 20 Leu Leu Ile Cys Asn Asp Gly Val Thr Ile Gln Ala Thr Leu Val Leu
 210 215 220
 Asp Ala Thr Gly Phe Ser Arg Ser Leu Val Gln Tyr Asp Lys Pro Tyr
 225 230 235 240
 25 Asn Pro Gly Tyr Gln Val Ala Tyr Gly Ile Leu Ala Glu Val Glu Glu
 245 250 255
 30 His Pro Phe Asp Val Asp Lys Met Leu Phe Met Asp Trp Arg Asp Ser
 260 265 270
 His Leu Asp Gln Asn Leu Glu Ile Lys Ala Arg Asn Ser Arg Ile Pro
 275 280 285
 35 Thr Phe Leu Tyr Ala Met Pro Phe Ser Ser Thr Arg Ile Phe Leu Glu
 290 295 300
 Glu Thr Ser Leu Val Ala Arg Pro Gly Leu Lys Met Glu Asp Ile Gln
 305 310 315 320
 40 Glu Arg Met Ala Tyr Arg Leu Lys His Leu Gly Ile Lys Val Lys Ser
 325 330 335
 45 Ile Glu Glu Asp Glu Arg Cys Val Ile Pro Met Gly Gly Pro Leu Pro
 340 345 350
 Val Leu Pro Gln Arg Val Leu Gly Ile Gly Gly Thr Ala Gly Met Val
 355 360 365
 50 His Pro Ser Thr Gly Tyr Met Val Ala Arg Thr Leu Ala Ala Ala Pro
 370 375 380
 Ile Val Ala Lys Ser Ile Ile Arg Tyr Leu Asn Asn Glu Lys Ser Met
 385 390 395 400
 55 Val Ala Asp Val Thr Gly Asp Asp Leu Ala Ala Gly Ile Trp Arg Glu
 405 410 415
 60 Leu Trp Pro Ile Glu Arg Arg Arg Gln Arg Glu Phe Phe Cys Phe Gly
 420 425 430
 Met Asp Ile Leu Leu Lys Leu Asp Leu Glu Gly Thr Arg Arg Phe Phe
 435 440 445

5

Asp Ala Phe Phe Asp Leu Glu Pro Arg Tyr Trp His Gly Phe Leu Ser
 450 455 460
 Ser Arg Leu Phe Leu Pro Glu Leu Val Thr Phe Gly Leu Ser Leu Phe
 465 470 475 480
 5 Gly His Arg Ser Asn Thr Cys Arg Val Glu Ile Met Ala Lys Gly Thr
 485 490 495
 Leu Pro Leu Ala Thr Met Ile Gly Asn Leu Val Arg Asp Arg Glu
 500 505 510
 10
 <210> 3
 <211> 991
 <212> DNA
 15 <213> *Tagetes erecta*
 <220>
 <221> CDS
 <222> (51)..(923)
 20 <223> beta-hydroxylase
 <400> 3
 ggcacgagat tgctgtccct tgtagctcaa gaccatttgg cttaggtcga atg cgg 56
 Met Arg
 1
 25
 tta ctt ggt cat aaa ccc aca acc ata act tgt cac ttc ccc ttt tct 104
 Leu Leu Gly His Lys Pro Thr Thr Ile Thr Cys His Phe Pro Phe Ser
 5 10 15
 30
 ttt tct atc aaa tca ttt acc cca att gtt agg ggc aga aga tgt act 152
 Phe Ser Ile Lys Ser Phe Thr Pro Ile Val Arg Gly Arg Arg Cys Thr
 20 25 30
 35
 gtt tgt ttt gtt gcc ggt ggc gac agt aat agt aac agt aat aat aat 200
 Val Cys Phe Val Ala Gly Asp Ser Asn Ser Asn Ser Asn Asn Asn
 35 40 45 50
 40
 agt gac agt aat agt aat aat ccg ggt ctg gat tta aac ccg gcg gtt 248
 Ser Asp Ser Asn Ser Asn Asn Pro Gly Leu Asp Leu Asn Pro Ala Val
 55 60 65
 45
 atg aac cgt aac cgt ttg gtt gaa gaa aaa atg gag agg aaa aaa tcg 296
 Met Asn Arg Asn Arg Leu Val Glu Glu Lys Met Glu Arg Lys Lys Ser
 70 75 80
 50
 gaa cga ttt act tat ctt gtt gca gct att atg tct act ttt gga att 344
 Glu Arg Phe Thr Tyr Leu Val Ala Ala Ile Met Ser Thr Phe Gly Ile
 85 90 95
 55
 act tca atg gcg gtt atg gcg gtt tat tac cgg ttt tca tgg caa atg 392
 Thr Ser Met Ala Val Met Ala Val Tyr Tyr Arg Phe Ser Trp Gln Met
 100 105 110
 60
 gag ggt gga gaa att cct tat gtg gag atg ttt ggt aca ttt gct ctc 440
 Glu Gly Gly Glu Ile Pro Tyr Val Glu Met Phe Gly Thr Phe Ala Leu
 115 120 125 130
 65
 tcc gtt ggt gct gcg gta gga atg gag tat tgg gca aga tgg gct cat 488
 Ser Val Gly Ala Ala Val Gly Met Glu Tyr Trp Ala Arg Trp Ala His
 135 140 145
 gag gca cta tgg cat gct tct ttg tgg cac atg cat gag tca cac cat 536
 Glu Ala Leu Trp His Ala Ser Leu Trp His Met His Glu Ser His His

	150	155	160	
	aag cca cga gaa ggt ccg ttt gag ctt aat gat gtg ttt gct ata aca	584		
	Lys Pro Arg Glu Gly Pro Phe Glu Leu Asn Asp Val Phe Ala Ile Thr			
	165 170 175			
5	aat gcg gtc ccg gcc att gcg ttg ctt agt tat ggg ttt ttc cac aaa	632		
	Asn Ala Val Pro Ala Ile Ala Leu Leu Ser Tyr Gly Phe Phe His Lys			
	180 185 190			
10	ggc ata att ccg ggt ctt tgt ttt ggg gcg gga ctg gga att acg gtg	680		
	Gly Ile Ile Pro Gly Leu Cys Phe Gly Ala Gly Leu Gly Ile Thr Val			
	195 200 205 210			
15	ttt gga atg gcg tat atg ttc gtc cac gac ggg cta gtt cac aga aga	728		
	Phe Gly Met Ala Tyr Met Phe Val His Asp Gly Leu Val His Arg Arg			
	215 220 225			
20	ttc caa gtg ggt ccg att gcg aat gtt ccc tat ctt cga agg gtt gca	776		
	Phe Gln Val Gly Pro Ile Ala Asn Val Pro Tyr Leu Arg Arg Val Ala			
	230 235 240			
	gcg gct cat cag ctg cat cac acg gaa aaa ttt aat ggt gtt cct tat	824		
	Ala Ala His Gln Leu His His Thr Glu Lys Phe Asn Gly Val Pro Tyr			
	245 250 255			
25	ggc ttg ttc ttg gga cct aag gag cta gaa gaa gtg ggt ggt acg gaa	872		
	Gly Leu Phe Leu Gly Pro Lys Glu Leu Glu Glu Val Gly Gly Thr Glu			
	260 265 270			
30	gaa ttg gac aag gag att caa aga aga att aaa ttg tat aat aat act	920		
	Glu Leu Asp Lys Glu Ile Gln Arg Arg Ile Lys Leu Tyr Asn Asn Thr			
	275 280 285 290			
35	aaa taaataaaatt ttgtataaaaa ttaatataat ttaatgatat ctttttgttt	973		
	Lys			
	taaaaaaaaa aaaaaaaaa	991		
40	<210> 4			
	<211> 291			
	<212> PRT			
	<213> Tagetes erecta			
45	<400> 4			
	Met Arg Leu Leu Gly His Lys Pro Thr Thr Ile Thr Cys His Phe Pro			
	1 5 10 15			
50	Phe Ser Phe Ser Ile Lys Ser Phe Thr Pro Ile Val Arg Gly Arg Arg			
	20 25 30			
	Cys Thr Val Cys Phe Val Ala Gly Gly Asp Ser Asn Ser Asn Ser Asn			
	35 40 45			
55	Asn Asn Ser Asp Ser Asn Ser Asn Asn Pro Gly Leu Asp Leu Asn Pro			
	50 55 60			
	Ala Val Met Asn Arg Asn Arg Leu Val Glu Glu Lys Met Glu Arg Lys			
	65 70 75 80			
60	Lys Ser Glu Arg Phe Thr Tyr Leu Val Ala Ala Ile Met Ser Thr Phe			
	85 90 95			
65	Gly Ile Thr Ser Met Ala Val Met Ala Val Tyr Tyr Arg Phe Ser Trp			
	100 105 110			

Gln Met Glu Gly Gly Glu Ile Pro Tyr Val Glu Met Phe Gly Thr Phe
 115 120 125
 Ala Leu Ser Val Gly Ala Ala Val Gly Met Glu Tyr Trp Ala Arg Trp
 130 135 140
 5 Ala His Glu Ala Leu Trp His Ala Ser Leu Trp His Met His Glu Ser
 145 150 155 160
 10 His His Lys Pro Arg Glu Gly Pro Phe Glu Leu Asn Asp Val Phe Ala
 165 170 175
 Ile Thr Asn Ala Val Pro Ala Ile Ala Leu Leu Ser Tyr Gly Phe Phe
 180 185 190
 15 His Lys Gly Ile Ile Pro Gly Leu Cys Phe Gly Ala Gly Leu Gly Ile
 195 200 205
 Thr Val Phe Gly Met Ala Tyr Met Phe Val His Asp Gly Leu Val His
 210 215 220
 20 Arg Arg Phe Gln Val Gly Pro Ile Ala Asn Val Pro Tyr Leu Arg Arg
 225 230 235 240
 25 Val Ala Ala Ala His Gln Leu His His Thr Glu Lys Phe Asn Gly Val
 245 250 255
 Pro Tyr Gly Leu Phe Leu Gly Pro Lys Glu Leu Glu Glu Val Gly Gly
 260 265 270
 30 Thr Glu Glu Leu Asp Lys Glu Ile Gln Arg Arg Ile Lys Leu Tyr Asn
 275 280 285
 Asn Thr Lys
 290
 35
 <210> 5
 <211> 1887
 <212> DNA
 40 <213> Tagetes erecta
 <220>
 <221> CDS
 <222> (141)..(1688)
 45 <223> epsilon-cyclase
 <400> 5
 ggcacgaggc aaagcaaagg ttgtttgttg ttgttggtga gagacactcc aatccaaaca 60
 50 gatacaaggc gtgactggat atttctctct cggttcctaac aacagcaacg aagaagaaaa 120
 agaatcatta ctaacaatca atg agt atg aga gct gga cac atg acg gca aca 173
 Met Ser Met Arg Ala Gly His Met Thr Ala Thr
 1 5 10
 55 atg gcg gct ttt aca tgc cct agg ttt atg act agc atc aga tac acg 221
 Met Ala Ala Phe Thr Cys Pro Arg Phe Met Thr Ser Ile Arg Tyr Thr
 15 20 25
 60 aag caa att aag tgc aac gct gct aaa agc cag cta gtc gtt aaa caa 269
 Lys Gln Ile Lys Cys Asn Ala Ala Lys Ser Gln Leu Val Val Lys Gln
 30 35 40
 65 gag att gag gag gaa gaa gat tat gtg aaa gcc ggt gga tcg gag ctg 317

	Glu	Ile	Glu	Glu	Glu	Glu	Asp	Tyr	Val	Lys	Ala	Gly	Gly	Ser	Glu	Leu	
	45						50					55					
	ctt	ttt	gtt	caa	atg	caa	cag	aat	aag	tcc	atg	gat	gca	cag	tct	agc	365
	Leu	Phe	Val	Gln	Met	Gln	Gln	Asn	Lys	Ser	Met	Asp	Ala	Gln	Ser	Ser	
5	60					65				70					75		
	cta	tcc	caa	aag	ctc	cca	agg	gta	cca	ata	gga	gga	gga	gga	gac	agt	413
	Leu	Ser	Gln	Lys	Leu	Pro	Arg	Val	Pro	Ile	Gly	Gly	Gly	Gly	Asp	Ser	
					80				85						90		
10	aac	tgt	ata	ctg	gat	ttg	gtt	gta	att	ggg	tgt	ggg	cct	gct	ggc	ctt	461
	Asn	Cys	Ile	Leu	Asp	Leu	Val	Val	Ile	Gly	Cys	Gly	Pro	Ala	Gly	Leu	
				95					100					105			
15	gct	ctt	gct	gga	gaa	tca	gcc	aag	cta	ggc	ttg	aat	gtc	gca	ctt	atc	509
	Ala	Leu	Ala	Gly	Glu	Ser	Ala	Lys	Leu	Gly	Leu	Asn	Val	Ala	Leu	Ile	
				110				115					120				
20	ggc	cct	gat	ctt	cct	ttt	aca	aat	aac	tat	ggg	gtt	tgg	gag	gat	gaa	557
	Gly	Pro	Asp	Leu	Pro	Phe	Thr	Asn	Asn	Tyr	Gly	Val	Trp	Glu	Asp	Glu	
		125					130					135					
25	ttt	ata	ggg	ctt	gga	ctt	gag	ggc	tgt	att	gaa	cat	gtt	tgg	cga	gat	605
	Phe	Ile	Gly	Leu	Gly	Leu	Glu	Gly	Cys	Ile	Glu	His	Val	Trp	Arg	Asp	
	140					145					150					155	
	act	gta	gta	tat	ctt	gat	gac	aac	gat	ccc	att	ctc	ata	ggg	cgt	gcc	653
	Thr	Val	Val	Tyr	Leu	Asp	Asp	Asn	Asp	Pro	Ile	Leu	Ile	Gly	Arg	Ala	
					160					165					170		
30	tat	gga	cga	gtt	agt	cgt	gat	tta	ctt	cac	gag	gag	ttg	ttg	act	agg	701
	Tyr	Gly	Arg	Val	Ser	Arg	Asp	Leu	Leu	His	Glu	Glu	Leu	Leu	Thr	Arg	
					175				180						185		
35	tgc	atg	gag	tca	ggc	gtt	tca	tat	ctg	agc	tcc	aaa	gtg	gaa	cgg	att	749
	Cys	Met	Glu	Ser	Gly	Val	Ser	Tyr	Leu	Ser	Ser	Lys	Val	Glu	Arg	Ile	
			190					195					200				
40	act	gaa	gct	cca	aat	ggc	cta	agt	ctc	ata	gag	tgt	gaa	ggc	aat	atc	797
	Thr	Glu	Ala	Pro	Asn	Gly	Leu	Ser	Leu	Ile	Glu	Cys	Glu	Gly	Asn	Ile	
		205					210					215					
45	aca	att	cca	tgc	agg	ctt	gct	act	gtc	gct	tct	gga	gca	gct	tct	gga	845
	Thr	Ile	Pro	Cys	Arg	Leu	Ala	Thr	Val	Ala	Ser	Gly	Ala	Ala	Ser	Gly	
	220					225					230					235	
	aaa	ctt	ttg	cag	tat	gaa	ctt	ggc	ggg	ccc	cgt	gtt	tgc	gtt	caa	aca	893
	Lys	Leu	Leu	Gln	Tyr	Glu	Leu	Gly	Gly	Pro	Arg	Val	Cys	Val	Gln	Thr	
				240					245						250		
50	gct	tat	ggg	ata	gag	gtt	gag	gtt	gaa	agc	ata	ccc	tat	gat	cca	agc	941
	Ala	Tyr	Gly	Ile	Glu	Val	Glu	Val	Glu	Ser	Ile	Pro	Tyr	Asp	Pro	Ser	
				255					260					265			
55	cta	atg	gtt	ttc	atg	gat	tat	aga	gac	tac	acc	aaa	cat	aaa	tct	caa	989
	Leu	Met	Val	Phe	Met	Asp	Tyr	Arg	Asp	Tyr	Thr	Lys	His	Lys	Ser	Gln	
			270					275					280				
60	tca	cta	gaa	gca	caa	tat	cca	aca	ttt	ttg	tat	gtc	atg	cca	atg	tct	1037
	Ser	Leu	Glu	Ala	Gln	Tyr	Pro	Thr	Phe	Leu	Tyr	Val	Met	Pro	Met	Ser	
		285					290					295					
65	cca	act	aaa	gta	ttc	ttt	gag	gaa	act	tgt	ttg	gct	tca	aaa	gag	gcc	1085
	Pro	Thr	Lys	Val	Phe	Phe	Glu	Glu	Thr	Cys	Leu	Ala	Ser	Lys	Glu	Ala	
	300					305					310					315	

	atg cct ttt gag tta ttg aag aca aaa ctc atg tca aga tta aag act	1133
	Met Pro Phe Glu Leu Leu Lys Thr Lys Leu Met Ser Arg Leu Lys Thr	
	320 325 330	
5	atg ggg atc cga ata acc aaa act tat gaa gag gaa tgg tca tat att	1181
	Met Gly Ile Arg Ile Thr Lys Thr Tyr Glu Glu Glu Trp Ser Tyr Ile	
	335 340 345	
10	cca gta ggt gga tcc tta cca aat acc gag caa aag aac ctt gca ttt	1229
	Pro Val Gly Gly Ser Leu Pro Asn Thr Glu Gln Lys Asn Leu Ala Phe	
	350 355 360	
15	ggt gct gct gct agc atg gtg cat cca gcc aca gga tat tcg gtt gta	1277
	Gly Ala Ala Ala Ser Met Val His Pro Ala Thr Gly Tyr Ser Val Val	
	365 370 375	
20	aga tca ctg tca gaa gct cct aat tat gca gca gta att gca aag att	1325
	Arg Ser Leu Ser Glu Ala Pro Asn Tyr Ala Ala Val Ile Ala Lys Ile	
	380 385 390 395	
25	tta ggg aaa gga aat tca aaa cag atg ctt gat cat gga aga tac aca	1373
	Leu Gly Lys Gly Asn Ser Lys Gln Met Leu Asp His Gly Arg Tyr Thr	
	400 405 410	
30	acc aac atc tca aag caa gct tgg gaa aca ctt tgg ccc ctt gaa agg	1421
	Thr Asn Ile Ser Lys Gln Ala Trp Glu Thr Leu Trp Pro Leu Glu Arg	
	415 420 425	
35	aaa aga cag aga gca ttc ttt ctc ttt gga tta gca ctg att gtc cag	1469
	Lys Arg Gln Arg Ala Phe Phe Leu Phe Gly Leu Ala Leu Ile Val Gln	
	430 435 440	
40	atg gat att gag ggg acc cgc aca ttc ttc cgg act ttc ttc cgc ttg	1517
	Met Asp Ile Glu Gly Thr Arg Thr Phe Phe Arg Thr Phe Phe Arg Leu	
	445 450 455	
45	ccc aca tgg atg tgg tgg ggg ttt ctt gga tct tcg tta tca tca act	1565
	Pro Thr Trp Met Trp Trp Gly Phe Leu Gly Ser Ser Leu Ser Ser Thr	
	460 465 470 475	
50	gac ttg ata ata ttt gcg ttt tac atg ttt atc ata gca ccg cat agc	1613
	Asp Leu Ile Ile Phe Ala Phe Tyr Met Phe Ile Ile Ala Pro His Ser	
	480 485 490	
55	ctg aga atg ggt ctg gtt aga cat ttg ctt tct gac ccg aca gga gga	1661
	Leu Arg Met Gly Leu Val Arg His Leu Leu Ser Asp Pro Thr Gly Gly	
	495 500 505	
60	aca atg tta aaa gcg tat ctc acg ata taaataactc tagtcgcgat	1708
	Thr Met Leu Lys Ala Tyr Leu Thr Ile	
	510 515	
65	cagtttagat tataggcaca tcttgcatat atatatgtat aaaccttatg tgtgctgtat	1768
	ccttacatca acacagtcac taattgtatt tcttggggta atgctgatga agtattttca	1828
	ggaaaaaaaaaaaaaaaa ctcgagacta gttcactctc tctctcctcg tgccgattc	1887
60	<210> 6	
	<211> 516	
	<212> PRT	
	<213> Tagetes erecta	
65	<400> 6	

10

Met Ser Met Arg Ala Gly His Met Thr Ala Thr Met Ala Ala Phe Thr
 1 5 10 15
 Cys Pro Arg Phe Met Thr Ser Ile Arg Tyr Thr Lys Gln Ile Lys Cys
 20 25 30
 5 Asn Ala Ala Lys Ser Gln Leu Val Val Lys Gln Glu Ile Glu Glu Glu
 35 40 45
 Glu Asp Tyr Val Lys Ala Gly Gly Ser Glu Leu Leu Phe Val Gln Met
 50 55 60
 10 Gln Gln Asn Lys Ser Met Asp Ala Gln Ser Ser Leu Ser Gln Lys Leu
 65 70 75 80
 15 Pro Arg Val Pro Ile Gly Gly Gly Gly Asp Ser Asn Cys Ile Leu Asp
 85 90 95
 Leu Val Val Ile Gly Cys Gly Pro Ala Gly Leu Ala Leu Ala Gly Glu
 100 105 110
 20 Ser Ala Lys Leu Gly Leu Asn Val Ala Leu Ile Gly Pro Asp Leu Pro
 115 120 125
 Phe Thr Asn Asn Tyr Gly Val Trp Glu Asp Glu Phe Ile Gly Leu Gly
 130 135 140
 25 Leu Glu Gly Cys Ile Glu His Val Trp Arg Asp Thr Val Val Tyr Leu
 145 150 155 160
 30 Asp Asp Asn Asp Pro Ile Leu Ile Gly Arg Ala Tyr Gly Arg Val Ser
 165 170 175
 Arg Asp Leu Leu His Glu Glu Leu Leu Thr Arg Cys Met Glu Ser Gly
 180 185 190
 35 Val Ser Tyr Leu Ser Ser Lys Val Glu Arg Ile Thr Glu Ala Pro Asn
 195 200 205
 Gly Leu Ser Leu Ile Glu Cys Glu Gly Asn Ile Thr Ile Pro Cys Arg
 210 215 220
 40 Leu Ala Thr Val Ala Ser Gly Ala Ala Ser Gly Lys Leu Leu Gln Tyr
 225 230 235 240
 45 Glu Leu Gly Gly Pro Arg Val Cys Val Gln Thr Ala Tyr Gly Ile Glu
 245 250 255
 Val Glu Val Glu Ser Ile Pro Tyr Asp Pro Ser Leu Met Val Phe Met
 260 265 270
 50 Asp Tyr Arg Asp Tyr Thr Lys His Lys Ser Gln Ser Leu Glu Ala Gln
 275 280 285
 Tyr Pro Thr Phe Leu Tyr Val Met Pro Met Ser Pro Thr Lys Val Phe
 290 295 300
 55 Phe Glu Glu Thr Cys Leu Ala Ser Lys Glu Ala Met Pro Phe Glu Leu
 305 310 315 320
 60 Leu Lys Thr Lys Leu Met Ser Arg Leu Lys Thr Met Gly Ile Arg Ile
 325 330 335
 Thr Lys Thr Tyr Glu Glu Glu Trp Ser Tyr Ile Pro Val Gly Gly Ser
 340 345 350
 65

Leu Pro Asn Thr Glu Gln Lys Asn Leu Ala Phe Gly Ala Ala Ala Ser
 355 360 365
 Met Val His Pro Ala Thr Gly Tyr Ser Val Val Arg Ser Leu Ser Glu
 370 375 380
 5 Ala Pro Asn Tyr Ala Ala Val Ile Ala Lys Ile Leu Gly Lys Gly Asn
 385 390 395 400
 Ser Lys Gln Met Leu Asp His Gly Arg Tyr Thr Thr Asn Ile Ser Lys
 405 410 415
 10 Gln Ala Trp Glu Thr Leu Trp Pro Leu Glu Arg Lys Arg Gln Arg Ala
 420 425 430
 15 Phe Phe Leu Phe Gly Leu Ala Leu Ile Val Gln Met Asp Ile Glu Gly
 435 440 445
 Thr Arg Thr Phe Phe Arg Thr Phe Phe Arg Leu Pro Thr Trp Met Trp
 450 455 460
 20 Trp Gly Phe Leu Gly Ser Ser Leu Ser Ser Thr Asp Leu Ile Ile Phe
 465 470 475 480
 Ala Phe Tyr Met Phe Ile Ile Ala Pro His Ser Leu Arg Met Gly Leu
 485 490 495
 25 Val Arg His Leu Leu Ser Asp Pro Thr Gly Gly Thr Met Leu Lys Ala
 500 505 510
 30 Tyr Leu Thr Ile
 515
 <210> 7
 <211> 1020
 35 <212> DNA
 <213> Tagetes erecta
 <220>
 <221> CDS
 40 <222> (101)..(796)
 <223> IPP isomerase
 <400> 7
 45 caggaattcg gcacgagctc aatctcaatc aaccctcttc ttctctccca gtatctatac 60
 caaaaacaac tcaaatctcc tccgtcgctc ttactccgcc atg ggt gac gac tcc 115
 Met Gly Asp Asp Ser
 1 5
 50 ggc atg gat gct gtt cag cga cgt ctc atg ttt aac gat gaa tgc att 163
 Gly Met Asp Ala Val Gln Arg Arg Leu Met Phe Asn Asp Glu Cys Ile
 10 15 20
 55 ttg gtg gat gag tgt gac aat gtg gtg gga cat gat acc aaa tac aat 211
 Leu Val Asp Glu Cys Asp Asn Val Val Gly His Asp Thr Lys Tyr Asn
 25 30 35
 tgt cac ttg atg gag aag att gaa aca ggt aaa atg ctg cac aga gca 259
 Cys His Leu Met Glu Lys Ile Glu Thr Gly Lys Met Leu His Arg Ala
 40 45 50
 60 ttc agc gtt ttt cta ttc aat tca aaa tac gag tta ctt ctt cag caa 307
 Phe Ser Val Phe Leu Phe Asn Ser Lys Tyr Glu Leu Leu Leu Gln Gln
 55 60 65
 65

	cgg tct gca acc aag gtg aca ttt cct tta gta tgg acc aac acc tgt	355
	Arg Ser Ala Thr Lys Val Thr Phe Pro Leu Val Trp Thr Asn Thr Cys	
	70 75 80 85	
5	tgc agc cat cca ctc tac aga gaa tcc gag ctt gtt ccc gaa aac gcc	403
	Cys Ser His Pro Leu Tyr Arg Glu Ser Glu Leu Val Pro Glu Asn Ala	
	90 95 100	
10	ctt gga gta aga aat gct gca cag agg aag ctg ttg gat gaa ctc ggt	451
	Leu Gly Val Arg Asn Ala Ala Gln Arg Lys Leu Leu Asp Glu Leu Gly	
	105 110 115	
15	atc cct gct gaa gat gtt ccc gtt gat cag ttt act cct tta ggt cgc	499
	Ile Pro Ala Glu Asp Val Pro Val Asp Gln Phe Thr Pro Leu Gly Arg	
	120 125 130	
20	atg ctc tac aag gct cca tct gat gga aag tgg gga gaa cat gaa ctt	547
	Met Leu Tyr Lys Ala Pro Ser Asp Gly Lys Trp Gly Glu His Glu Leu	
	135 140 145	
25	gat gaa gtg gcg gat atc aaa tat gtg aac caa gaa gag tta aag gag	595
	Asp Tyr Leu Leu Phe Ile Val Arg Asp Val Ala Val Asn Pro Asn Pro	
	150 155 160 165	
30	gat gaa gtg gcg gat atc aaa tat gtg aac caa gaa gag tta aag gag	643
	Asp Glu Val Ala Asp Ile Lys Tyr Val Asn Gln Glu Glu Leu Lys Glu	
	170 175 180	
35	ctg cta agg aaa gca gat gcg ggg gag gag ggt ttg aag ctg tct cca	691
	Leu Leu Arg Lys Ala Asp Ala Gly Glu Glu Gly Leu Lys Leu Ser Pro	
	185 190 195	
40	tgg ttc agg tta gtg gtt gat aac ttc ttg ttc aag tgg tgg gat cat	739
	Trp Phe Arg Leu Val Val Asp Asn Phe Leu Phe Lys Trp Trp Asp His	
	200 205 210	
45	gtg caa aag gtt aca ctc act gaa gca att gat atg aaa acc ata cac	787
	Val Gln Lys Val Thr Leu Thr Glu Ala Ile Asp Met Lys Thr Ile His	
	215 220 225	
50	aag ctg ata tagaaacaca ccctcaaccg aaaagttcaa gcctaataat	836
	Lys Leu Ile	
	230	
55	tcggggttggg tcgggtctac catcaattgt ttttttcttt taagaagttt taatctctat	896
	ttgagcatgt tgattcttgt cttttgtgtg taagattttg ggtttcgttt cagttgtaat	956
	aatgaaccat tgatggtttg caattttcaag ttcctatcga caaaaaaaaaa aaaaaaaaaa	1016
60	actc	1020
	<210> 8	
	<211> 232	
	<212> PRT	
	<213> Tagetes erecta	
	<400> 8	
	Met Gly Asp Asp Ser Gly Met Asp Ala Val Gln Arg Arg Leu Met Phe	
	1 5 10 15	
	Asn Asp Glu Cys Ile Leu Val Asp Glu Cys Asp Asn Val Val Gly His	
	20 25 30	
65		

[illegible]

CONFIDENTIAL

THIS PAGE BLANK (USPTO)

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



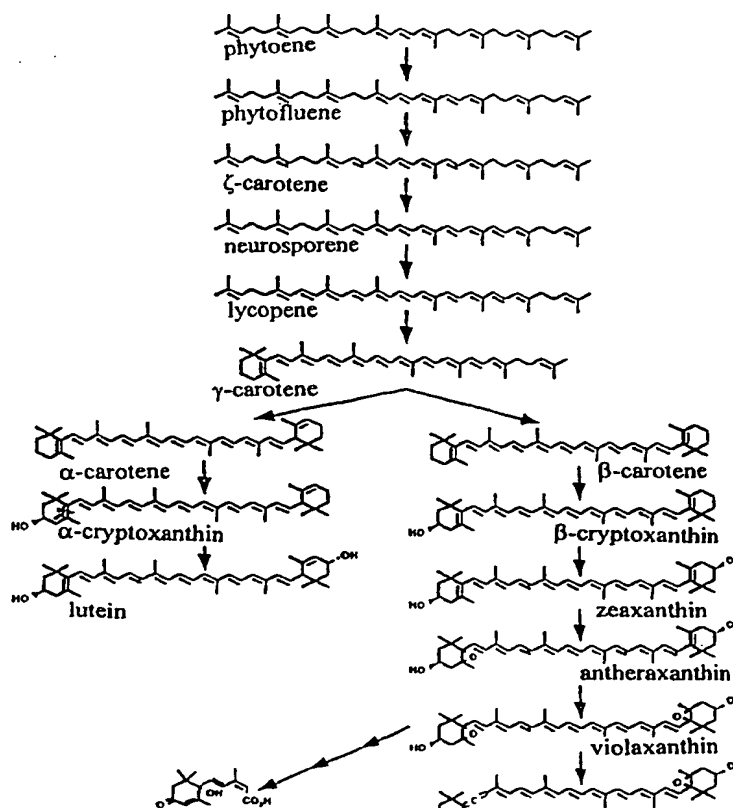
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 15/53, 15/61, 15/11, 9/02, 9/90, A01H 5/00, C12N 15/82, 5/10	A3	(11) International Publication Number: WO 00/32788
		(43) International Publication Date: 8 June 2000 (08.06.00)
(21) International Application Number: PCT/DK99/00668 (22) International Filing Date: 30 November 1999 (30.11.99) (30) Priority Data: 09/201,641 30 November 1998 (30.11.98) US (71) Applicant (for all designated States except US): CHR. HANSEN A/S [DK/DK]; Boege Allé 10-12, P.O. Box 407, DK-2970 Hoersholm (DK). (72) Inventors; and (75) Inventors/Applicants (for US only): DELLAPENNA, Dean [US/US]; 4135 Longknife Road, Reno, NV 89557 (US). CUNNINGHAM, Francis, X. [US/US]; 2727 Washington Avenue, Chevy Chase, MD 20815 (US). (74) Agent: PLOUGMANN, VINGTOFT & PARTNERS A/S; Sankt Annæ Plads 11, P.O. Box 3007, DK-1021 Copen- hagen K (DK).		(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), DM, EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR (Utility model), KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published With international search report. (88) Date of publication of the international search report: 5 October 2000 (05.10.00)

(54) Title: METHOD FOR REGULATING CAROTENOID BIOSYNTHESIS IN MARIGOLDS

(57) Abstract

A method for manipulating the ratio of various carotenoids in a plant as a means for augmenting the accumulation of selected carotenoids is described. Transgenic marigold plants which produce various ratios of carotenoids and methods for producing the same are described. Preferably, various carotenoids are accumulated in the petals of marigold by selecting a specific combination of isolated DNAs encoding various enzymes involved in the carotenoid biosynthesis pathway to produce antisense RNA, sense RNA or combinations thereof. Transgenic marigold which specifically accumulates carotenoids in the petals are described. Also described are isolated DNA sequences encoding the marigold genes beta-cyclase, epsilon-cyclase, beta-hydroxylase and isopentyl pyrophosphate isomerase.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 99/00668

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/53 C12N15/61 C12N15/11 C12N9/02 C12N9/90
A01H5/00 C12N15/82 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 36998 A (UNIVERSITY OF MARYLAND COLLEGE PARK, USA) 9 October 1997 (1997-10-09)	41-47
Y	the whole document	5-16, 20-40
Y	CUNNINGHAM, F.X., JR., ET AL.: "Functional analysis of the beta and epsilon lycopene cyclase enzymes of Arabidopsis reveals a mechanism for control of cyclic carotenoid formation" THE PLANT CELL, vol. 8, September 1996 (1996-09), pages 1613-1626, XP000881768 the whole document	1-4, 9-19, 26-32, 36-38

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

31 May 2000

Date of mailing of the international search report

20.05.00

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040 Telex 31 651 epo nl

Authorized officer

INTERNATIONAL SEARCH REPORT

Int. l. Application No

PCT/DK 99/00668

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96 28014 A (YISSUM RES DEV CO ;HIRSCHBERG JOSEPH (IL); CUNNINGHAM FRANCIS XAVI) 19 September 1996 (1996-09-19) page 5, line 2 -page 6, line 6 page 16, line 8 -page 16, line 21 ---	1-4, 13-19, 26-32, 36-38
Y	WO 96 36717 A (CENTRE NAT RECH SCIENT ;KUNTZ MARCEL (GB)) 21 November 1996 (1996-11-21) page 1, line 5 -page 14, line 5 ---	1-4, 13-19, 26-32, 36-38
Y	HIRSCHBERG, J. ET AL., : "Molecular genetics of the carotenoid biosynthesis pathway in plants and algae" PURE & APPL. CHEM., vol. 69, no. 10, 1997, pages 2151-2158, XP000872988 the whole document ---	1-32, 36-38
Y	BIRD, C.R. ET AL.: "Using antisense RNA to study gene function: inhibition of carotenoid biosynthesis in transgenic tomatoes" BIO/TECHNOLOGY, vol. 9, 1991, pages 635-639, XP002131518 the whole document ---	26-32, 36-38
Y	SUN Z ET AL: "Cloning and functional analysis of the beta-carotene hydroxylase of Arabidopsis thaliana" JOURNAL OF BIOLOGICAL CHEMISTRY,US,AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 271, no. 40, 4 October 1996 (1996-10-04), pages 24349-24352, XP002121880 ISSN: 0021-9258 the whole document ---	5-8, 13-16, 20-22
Y	WO 96 02594 A (IND ORGANICA S A DE C V) 1 February 1996 (1996-02-01) page 1, line 9 -page 1, line 22 ---	1-40
	--- -/--	

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/DK 99/00668

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>CUNNINGHAM F X ET AL: "GENES AND ENZYMES OF CAROTENOID BIOSYNTHESIS IN PLANTS" ANNUAL REVIEW OF PLANT PHYSIOLOGY AND PLANT MOLECULAR BIOLOGY, XX, ANNUAL REVIEWS INC, vol. 49, 1998, pages 557-583, FIGURES, XP000881772 ISSN: 1040-2519 page 560, paragraph 3 page 572, paragraph 2 -page 573, paragraph 2; figures</p>	1-40
A	<p style="text-align: center;">---</p> <p>GOMEZ R ET AL: "CAROTENOIDS FROM MARIGOLD (TAGETES ERECTA) PETALS AND THEIR ESTERIFIED FATTY ACIDS" REVISTA ESPANOLA DE FISILOGIA, ES, ASOCIACION REVISTA ESPANOLA DE FISILOGIA, PAMPLONA, vol. 34, no. 3, 1978, pages 253-256, XP000881763 ISSN: 0034-9402 the whole document</p>	
A	<p style="text-align: center;">---</p> <p>KOTHARIN, S.L., CHANDRA, N.: "PLANT REGENERATION IN CALLUS SUSPENSION CULTURES OF TAGETES-ERECTA AFRICAN MARIGOLD" JOURNAL OF PLANT PHYSIOLOGY, vol. 122, 1986, pages 235-242, XP000882001 the whole document</p>	
A	<p style="text-align: center;">---</p> <p>WO 98 06862 A (SHEWMAKER CHRISTINE K ;CALGENE INC (US)) 19 February 1998 (1998-02-19) page 2, line 6 -page 2, line 31 page 7, line 2 -page 13, line 5 page 24, line 22 -page 25, line 4</p>	
E	<p style="text-align: center;">---</p> <p>WO 99 63055 A (CUNNINGHAM FRANCIS X JR ;UNIV MARYLAND (US); SUN ZAIREN (US)) 9 December 1999 (1999-12-09) figure 21A</p>	41-47
E	<p style="text-align: center;">---</p> <p>WO 99 61652 A (UNIV MARYLAND ;CUNNINGHAM FRANCIS X (US)) 2 December 1999 (1999-12-02) the whole document</p> <p style="text-align: center;">-----</p>	35,40

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 99/00668

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-4, 17-19 (all complete); 13-16, 26-40 (all partially)

DNA sequence encoding marigold beta-cycalse; transgenic plant material containing said DNA; transgenic plant material containing DNA which produces an RNA that is antisense to marigold beta-cyclase; method for manipulating carotenoid synthesis in a plant material

2. Claims: 5-8, 20-22 (all complete); 13-16, 26-40 (all partially)

DNA sequence encoding marigold beta-hydroxylase; transgenic plant material containing said DNA; transgenic plant material containing DNA which produces an RNA that is antisense to marigold beta-hydroxylase; method for manipulating carotenoid synthesis in a plant material

3. Claims: 9-12, 23-25 (all complete); 13-16, 26-40 (all partially)

DNA sequence encoding marigold epsilon-cycalse; transgenic plant material containing said DNA; transgenic plant material containing DNA which produces an RNA that is antisense to marigold epsilon-cyclase; method for manipulating carotenoid synthesis in a plant material

4. Claims: 41-47 (all complete); 29, 31-40 (all partially)

DNA sequence encoding marigold IPP isomerase; transgenic plant material containing said DNA; transgenic plant material containing DNA which produces an RNA that is antisense to marigold IPP isomerase; method for manipulating carotenoid synthesis in a plant material

5. Claims: 13, 26, 29-31, 33-40 (all partially)

Transgenic plant material containing DNA sequence encoding marigold epsilon-hydroxylase; transgenic plant material containing DNA which produces an RNA that is antisense to marigold epsilon-hydroxylase; method for manipulating carotenoid synthesis in a plant material

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. lional Application No

PCT/DK 99/00668

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9736998	A	09-10-1997	US 5744341 A	28-04-1998
			AU 1578497 A	22-10-1997
			BR 9708375 A	03-08-1999
			CA 2250096 A	09-10-1997
			EP 0889952 A	13-01-1999
WO 9628014	A	19-09-1996	US 5792903 A	11-08-1998
			AU 5093196 A	02-10-1996
			CA 2214469 A	19-09-1996
			EP 0820221 A	28-01-1998
WO 9636717	A	21-11-1996	AU 5897796 A	29-11-1996
WO 9602594	A	01-02-1996	US 5523494 A	04-06-1996
			AU 2682095 A	16-02-1996
			DE 69509069 D	20-05-1999
			DE 69509069 T	05-08-1999
			EP 0741795 A	13-11-1996
			ES 2103692 T	01-10-1997
WO 9806862	A	19-02-1998	AU 4058497 A	06-03-1998
			CN 1227609 A	01-09-1999
			EP 0925366 A	30-06-1999
WO 9963055	A	09-12-1999	AU 4410999 A	20-12-1999
WO 9961652	A	02-12-1999	AU 4184699 A	13-12-1999

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

☐ BLACK BORDERS

☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES

☐ FADED TEXT OR DRAWING

☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING

☐ SKEWED/SLANTED IMAGES

☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS

☐ GRAY SCALE DOCUMENTS

☐ LINES OR MARKS ON ORIGINAL DOCUMENT

☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY

☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)